

Analysis of CMV-specific T cell responses and CMV-associated changes in the ageing human immune system

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1 Abstract

Ageing of the immune system, also called immunosenescence, is a phenomenon that leads to increased susceptibility to infections in elderly people. Persistent cytomegalovirus (CMV) infection induces strong T cell responses in humans and is thought to be one of the driving forces of immunosenescence.

In this work CMV-induced alterations in the T cell compartment of human individuals were analysed in terms of frequencies and absolute numbers per ml blood. Peripheral blood mononuclear cells (PBMC) from 50 donors in three different age groups were stimulated *in vitro* and examined for the phenotypic markers CD45RA and CD27 and the effector molecules CD40L, IFN γ , IL2 and TNF by polychromatic flow cytometry. For this purpose, an 11-colour flow cytometric panel was established.

The results showed that the memory T cell subset distribution became further differentiated with advancing age of the donor. Further differentiated T cells expressed less CD3, CD4 and CD8 on their surface than T_N cells.

The frequency of responding polyfunctional T cells to stimulation with OKT3 or CMV peptide pools phosphoprotein 65 (pp65) or immediate-early protein1 (IE1) increased with the age of the donor. The memory subset distribution differed between CMV-seronegative and CMV-seropositive donors and was correlated with the response size in the CMV-seropositive group. CMV infection with large CMV-specific T cell responses resulted in further differentiated T cell subsets.

In addition, CMV infection appeared to affect unrelated immune responses. The results showed that CMV-infected donors had higher numbers of activated T cells following stimulation with OKT3 or purified protein derivate (PPD) from *Mycobacterium tuberculosis*. Although the cytokine profile of responding T cells appeared to be very similar in CMV-negative and CMV-positive individuals, CMV-infected donors had more highly differentiated T cells.

Polyfunctional T cells expressed quantitatively and qualitatively more activation marker on a per cell level than monofunctional cells and therefore appeared to be more effective.

Furthermore, polyfunctional T cells expressed reduced amounts of surface CD3, CD4 and CD8 compared to monofunctional or non-responding T cells.

To examine the effect of the immunosuppressive drugs cyclosporine A (CSA) and tacrolimus (FK506) on CMV-specific T cells, the activation molecules CD40L, IFN γ , IL2, TNF and CD107a were analysed. *In vitro* incubation of PBMC with CSA and FK506 did not alter the frequency of T cells responding to pp65 but inhibited the expression of CD40L, IFN γ , IL2 and TNF. Degranulation of pp65-specific T cells was not affected.

In summary, the results indicate that CMV has a significant influence on the immune system of healthy people. The memory subset composition of the entire T cell compartment depends on how the immune system responds to CMV (large or small responses) rather than the presence or absence of infection. Irrespective of response size or age a robust population of polyfunctional CMV-specific T cells is present and may be in control of CMV.

2 Zusammenfassung

Die Veraenderungen des Immunsystems mit zunehmendem Alter, auch Immunseneszenz genannt, resultieren in erhoelter Infektanfelligkeit. Infolge dessen leiden aeltere Menschen unter haeufigeren und schwerer verlaufenden Infekten, Autoimmunerkrankungen und vermindertem Impfschutz. Immunseneszenz entsteht nicht nur aufgrund chronologischen Alterns, sondern wird auch durch andere teils ungeklaerte Faktoren verursacht. Cytomegalievirus (CMV) ist ein Herpesvirus, dass nach primaerer Infektion in immunkompetenten Menschen lebenslang persistiert. Infolge chronischer Immunaktivierung traegt CMV zur beschleunigten Immunseneszenz bei.

In dieser Arbeit wurden die Alters- und CMV-induzierten Veraenderungen in humanen T-Zellen analysiert. Dafuer wurden PBMC von 50 gesunden Spendern in drei verschiedenen Altersgruppen antigenspezifisch (CMV-Proteine pp65 und IE1 und protein purified derivate von *Mycobacterium tuberculosis* (PPD)) und polyklonal (OKT3) *in vitro* stimuliert. Ein 11-Farben-Panel wurde etabliert, um die simultane Expression der Differenzierungsmarker CD45RA und CD27 und der Aktivierungsmarker CD40L, IFN γ , IL2 und TNF auf T-Zellen durchflusszytometrisch zu untersuchen. Die Ergebnisse wurden als Absolutwerte in Zellen pro ml Blut und als Prozent der jeweiligen Referenzpopulation analysiert.

Die Ergebnisse dieser Arbeit zeigten, dass die antigenabhaengige Differenzierung der Gedaechnis-T-Zellen mit zunehmendem Alter und CMV-Infektion zunahm. Die Differenzierung der Gedaechnis-T-Zellen in CMV-positiven Spendern war ausserdem mit der Antwortgrosse gegen das CMV-Protein pp65 korreliert. Aeltere Spender mit grossen CMV-spezifischen T-Zell-Antworten hatten die am weitesten differenzierten T-Zellen. Differenzierte T-Zellen zeigten eine Herabregulation der Oberflaechenmolekuele CD3, CD4 und CD8 verglichen mit naiven T-Zellen.

Die pp65-spezifische, aber nicht die IE1-spezifische T-Zell-Antwort nahm mit zunehmendem Alter der Probanden zu. Diese T-Zell-Antworten waren mit zunehmender Grosse auch zunehmend polyfunktional, das heisst, sie produzierten mehrere der untersuchten Aktivierungsmarker gleichzeitig. Polyfunktionale T-Zellen nach antigenspezifischer und nach polyklonaler Stimulation exprimierten qualitativ und quantitativ mehr Aktivierungsmarker pro

T-Zelle und zeigten eine Herabregulation der Oberflaechenmolekuele CD3, CD4 und CD8. Dies deutet auf eine staerkere Aktivierung der polyfunktionalen T-Zellen hin.

Desweiteren konnte gezeigt werden, dass CMV-Infektion einen Einfluss auf andere T-Zell-Antworten hat. Die T-Zell-Antworten nach antigenspezifischer (PPD) und polyklonaler (OKT3) Stimulation waren groesser in CMV-infizierten als in nichtinfizierten Probanden. Dieser Anstieg war bedingt durch mehr aktivierte T_{EM}-Zellen in CMV-infizierten Personen.

Zusaetzlich wurde der Effekt der immunsupprimierenden Medikamente CyclosporinA (CSA) und Takrolimus (FK506) auf polyfunktionale CMV-spezifische T-Zellen untersucht. Die *in vitro* Inkubation von PBMC mit CSA und FK506 resultierte in einem massiven Verlust von polyfunktionalen T-Zellen nach Stimulation mit pp65. In pp65-spezifischen T-Zellen inkubiert mit CSA oder FK506 konnte nur Degranulation nachgewiesen werden, aber keine anderen Aktivierungsmarker.

Zusammenfassend kann gesagt werden, dass CMV einen grossen Einfluss auf das Immunsystem gesunder Individuen hat. Die Differenzierung der Gedaechnis-T-Zellen ist nicht nur abhaengig von der Infektion mit CMV, sondern auch davon, wie das Immunsystem auf CMV reagiert. Polyfunktionale CMV-spezifische T-Zellen, die wahrscheinlich CMV kontrollieren, sind vorhanden und werden nicht von dysfunktionalen T-Zellen im Alter verdraengt.

3 Abbreviations

aa	Amino acids
Ab	Antibody
AIDS	Aquired immune deficiency syndrome
APC	Antigen presenting cell
APC	Allophycocyanin
APC-Cy7	Allophycocyanin cyanin7
APC-H7	Allophycocyanin cyanin dye
Aquid	Aqua viability dye
ATG	Anti-thymocyte globulin
BCG	Bacillus Calmette-Guérin, vaccine prepared from <i>Mycobacterium bovis</i> ,
BFA	Brefeldin A
BSA	Bovine serum albumine
CBA	Cytokine bead array
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CD40L	CD40-ligand
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence intervall
CMV	Cytomegalovirus
CSA	Cyclosporin A
CTL	Cytotoxic T lymphocyte
DMSO	Dimethyl sulfoxide
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FK506	Tacrolimus
FMO control	Fluorescence minus one control
FSC	Forward scatter

g	Centrifugal force
h	Hour
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
ICS	Intracellular cytokine staining
IE	Immediate early protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRP	Immune risk profile
IQR	Interquartile range
LAMP	Lysosomal-associated membrane protein
LPS	Lipopolysaccharide
M. tuberculosis	Mycobacterium tuberculosis
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
min	Minute
MIP	Macrophage inflammatory protein
ng	Nanogram
NK cells	Natural killer cells
PB	Pacific blue
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PECy7	Phycoerythrin cyanine dye
PerCP	Peridinin chlorophyll protein
PFA	Paraformaldehyde
PMT	Photomultiplier
pp65	Phosphoprotein 65
PPD	Purified protein derivate of <i>M. tuberculosis</i>
Qdot605	Quantum Dot 605
R _s	Spearman's correlation coefficient
ROC curve	Receiver operating characteristics curve

RPMI medium	Roswell Park Memorial Institute Medium
RT	Room temperature
SEB	Staphylococcal enterotoxin B
SEM	Standard error of mean
SSC	Sideward scatter
Std	Standard deviation
T _{CM} cell	Central memory T cell
TCR	T cell receptor
T _{EM} cell	Effector memory T cell
T _{EMRA} cell	Effector memory revertant T cell
T _N cell	Naïve T cell
TNF	Tumour necrosis factor
T _{reg}	Regulatory T cell
µg	Mikrogram
µl	Mikroliter

4 Introduction

4.1 Immune system

Humans are constantly surrounded by potentially harmful infectious agents. The immune system has evolved to protect the host against these pathogens (bacteria, viruses, fungi and parasites). The immune system is composed of many different cell types and can be divided into two parts: the innate (non-specific) immune response and the adaptive (specific) immune response. Both immune responses can distinguish between self and non-self antigens but the effector functions and mechanisms are different. While the innate immune response is based on the recognition of conserved features, the adaptive immune response is specific to the invading agent. Nonetheless, the innate and adaptive immune responses do not operate in isolation and are dependent on each other for efficient and long-lasting protection (Figure 1).

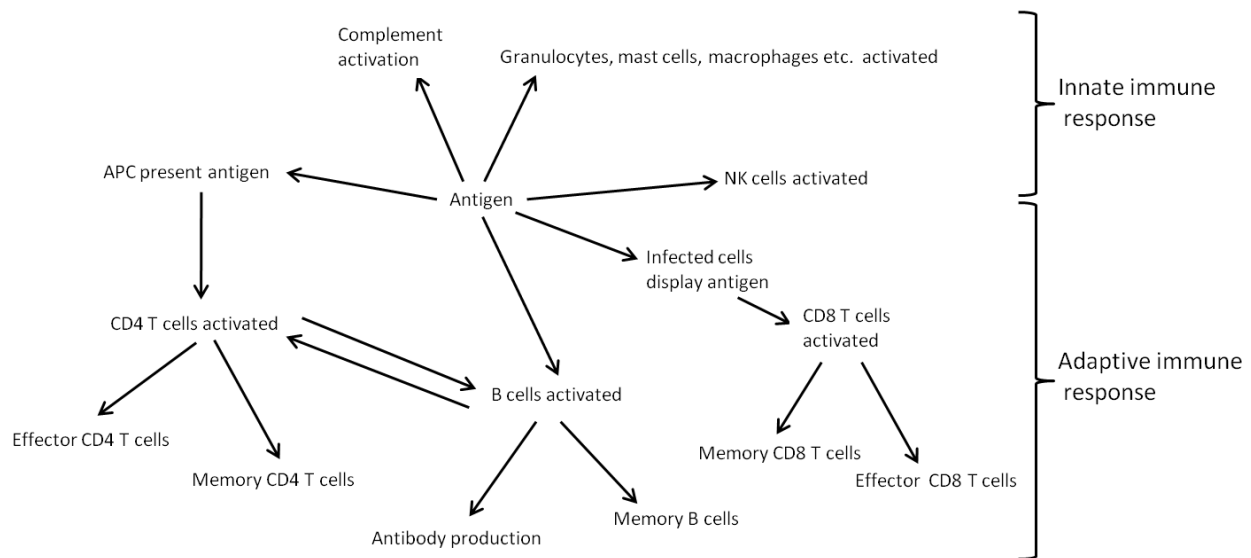


Figure 1: **Schematic view of the human immune response to non-self antigen.**

4.1.1 Innate immunity

Innate immunity evolved very early during evolution and is present in all multicellular organisms. It consists of soluble factors, such as complement proteins and a diversity of cells including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells [1]. Innate immunity involves the interactions of these cells with each other through cell surface receptors and soluble molecules that act non-specifically.

Innate immunity is used to rapidly generate an initial response against pathogens, either eliminating it or containing it until the adaptive immune response can develop. A second exposure to the same antigen provokes the same immune response as the first encounter. The advantage is that the pathogens can be immediately attacked every time they invade the host. Innate immunity limits pathogen entry, induces local inflammatory responses, prevents the establishment of infection and clears both host and microbial debris.

4.1.2 Adaptive immunity

Adaptive immunity is only found in vertebrates and refers to antigen-specific defence mechanisms that take several days to become protective [1]. The adaptive immune response is maintained by highly specific lymphocytes called B cells (humoral immune response) and T cells (cell-mediated immune response). Each lymphocyte clone expresses cell surface receptors of a single specificity. After specific activation these cells proliferate and form clones of cells with identical surface receptor specificity. The repertoire of receptor specificities is very diverse because it is encoded by genes that undergo random somatic rearrangement during lymphocyte development [2]. The downside to the specificity of adaptive immunity is that only a very few B and T cells in the body recognise a given antigenic determinant (epitope). These few cells then must rapidly proliferate in order to produce enough cells to mount an effective immune response against that particular epitope, which takes a few days. Binding of the receptor to the complementary antigen is required to activate the cell. B cells are activated by antigen that binds directly to the B cell receptor. Activated B cells differentiate into plasma cells, which secrete antibodies specific for the antigen. Only a few activated B cells will survive the subsequent contraction phase and differentiate to long term memory B cells. These memory B cells allow the mounting of a fast adaptive immune response to a second encounter with the same antigen. T cells can only be activated by antigen that is processed and presented by antigen-presenting cells (APC). The antigens are presented to T cells as a peptide bound to the major histocompatibility complex (MHC) of the APC.

4.2 T cells

4.2.1 Classification of T cells by lineage markers

T cells are a major part of the adaptive immune system and are characterised by the expression of the T cell receptor (TCR). The peripheral T cell pool is formed from thymic output and

includes T cells with various differentiation stages and functional properties. The peripheral T cell pool can be divided into smaller subsets based on the expression of surface molecules and the properties of single cells. T cells can be distinguished by the type of TCR expressed, with about 90% of T cells expressing an $\alpha\beta$ -receptor and a minority of T cells expressing the $\gamma\delta$ -receptor [3]. T cells with the $\gamma\delta$ -TCR have been characterised to a lesser extent and will not be considered in this work. T cells expressing the $\alpha\beta$ -TCR can be further divided regarding the expression of the accessory molecules, CD4 and CD8, on the cell surface. CD4 T cells recognise MHC class II which are expressed on APC and CD8 T cells recognising MHC class I molecules which are expressed on most nucleated cells [4]. CD8 T cells are also called cytotoxic T cells (CTL) and are responsible for killing tumour and infected host cells by the secretion of cytokines and perforin- and granzyme-mediated cytotoxicity [5]. CD4 T cells can be further differentiated into several subpopulations depending on their surface markers and their ability to produce cytokines and chemokines. The majority of CD4 T cells secrete cytokines which promote the activation of professional APC (e.g. macrophages, dendritic cells), B cells and CD8 T cells. A small subset of CD4 T cells can dampen the immune response and are therefore called regulatory T cells (T_{reg}) [6]. Some CD4 T cells exhibit cytotoxic functions and can lyse other host cells [7, 8].

4.2.2 Classification of T cells by phenotype and differentiation

T cells that have been released from the thymus and not yet encountered their cognate antigen are termed naive T (T_{N}) cells until they come across their antigen for the first time. T_{N} cells are the most homogenous subpopulation of T cells, lacking most effector functions. The binding of the specific antigen, presented by an APC to a TCR, and costimulatory signals lead to T cell activation, clonal expansion and the generation of effector T cells. Short-lived effector T cells migrate to peripheral tissues and display immediate effector functions early during primary infection [9]. After clearance of the antigen most effector T cells die and only a few will remain to protect the body against recurrence with the same pathogen [10]. These long-lived memory T cells provoke a faster immune response after new antigen encounter than their T_{N} cell precursors [11]. Both effector and memory T cells need lower levels of TCR engagement and costimulation than T_{N} cells, which enable them to eliminate pathogens faster. Memory T cells can be divided into two major subpopulations, central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). T_{CM} cells express lymph node-homing receptors, efficiently stimulate APC, lack immediate effector functions but differentiate to effector T cells after proliferation. In contrast, T_{EM} cells

migrate to inflamed peripheral tissues and display immediate effector functions [12]. Terminally differentiated effector memory T cells (T_{EMRA}) have little proliferative capacity and display potent effector functions [13]. Some markers and effector functions which distinguish between T_N , T_{CM} , T_{EM} and T_{EMRA} cells are shown in Figure 2.

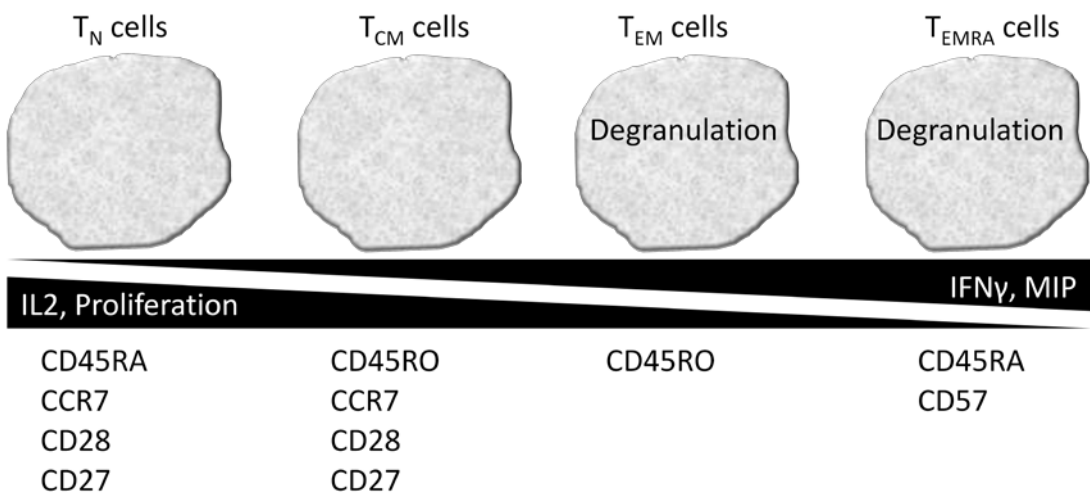


Figure 2: **T cell subsets.** The four memory T cell subsets with their functions (in black) and phenotypes (below) are shown.

Considerable knowledge about T cell differentiation has been drawn from longitudinal studies in mouse models. Longitudinal studies in humans are, for obvious reasons, more difficult to perform. Therefore, researchers have mainly studied different T cell subsets in the peripheral blood. One of the first published works on this topic by Hamann *et al.* (1997) distinguished between T_N cells, memory T cells with high proliferative capacity and effector T cells which were cytotoxic on the basis of phenotypic markers [14]. Since then, several models of T cell differentiation have been suggested (Figure 3). Hamann *et al.* (1997) proposed a differentiation model based on the markers CD27 and CD45RA [15]. First, CD8 T_N cells lose CD45RA, undergo extensive expansion and afterwards lose CD27. Fully differentiated effector T cells which no longer proliferate then re-express CD45RA (Figure 3a). CD27 belongs to the tumor necrosis factor (TNF) superfamily and binds to CD70 on the surface of dendritic cells during activation [16]. This interaction results in an irreversible loss of CD27 on CD4 and CD8 T cells [17]. CD45R isoforms regulate signalling through the TCR-complex. T cell activation leads to a reversible shift from the CD45RA isoform to the alternatively spliced isoform of CD45RO (rev. in [18, 19]). Another model has suggested that the differentiation of T cells depends on the cytokines and can result in effector and T_{EM} cells or in non-cytotoxic cells which differentiate

into T_{CM} cells (Figure 3b) [20]. A third model has proposed that T_N cells develop into effector cells which then can differentiate into either T_{EM} or T_{CM} cells and eventually to T_{EMRA} cells (Figure 3c) [21]. Wherry *et al.* (2003) proposed a linear model where T cells develop from T_N to effector to T_{EM} and then develop into T_{CM} cells (Figure 3d) [22]. The “stem-cell like plasticity”-model proposes that every T_N cell has the potential to develop into any of the major T cell subsets (Figure 3e) [9]. The differentiation pathway of T cells and the role of the subsets are still not fully resolved.

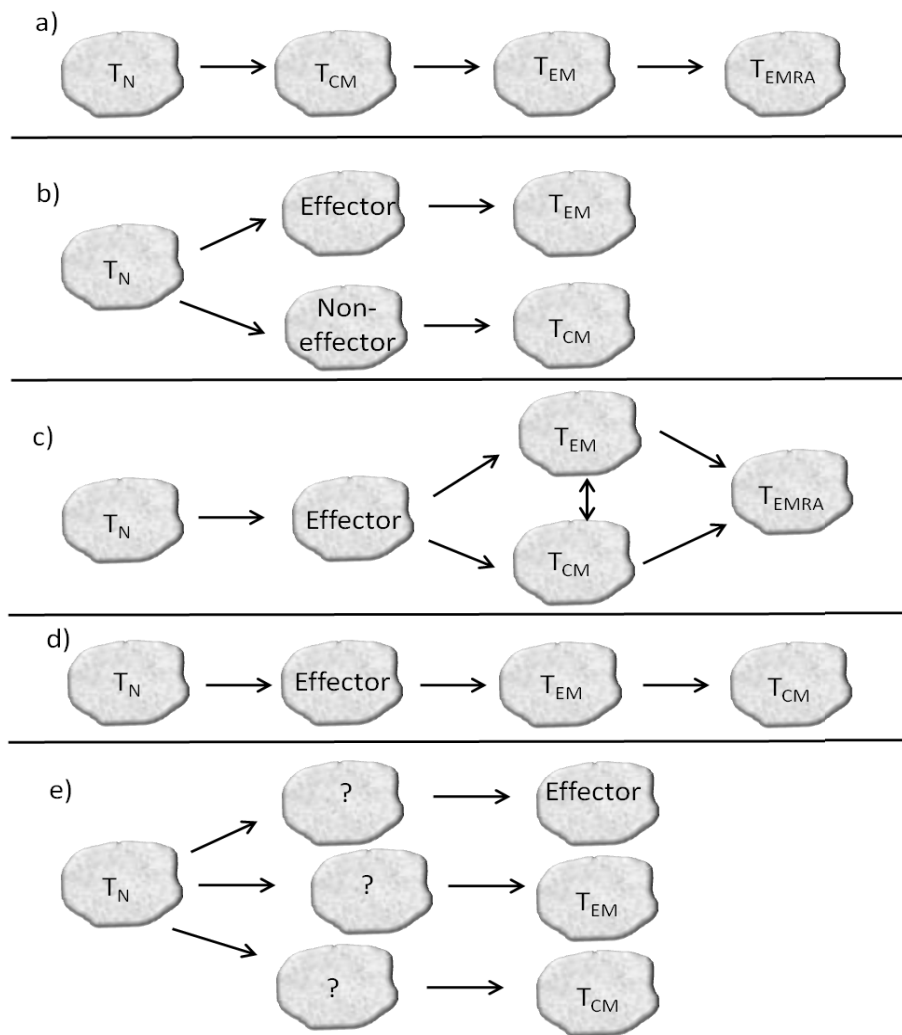


Figure 3: **Different models of T cell differentiation in humans.**

In this study the phenotype markers CD45RA and CD27 were used to distinguish between T_N , T_{CM} , T_{EM} and T_{EMRA} cells according to the model proposed by Hamann *et al.* (1997) as shown in Figure 3 [14, 15].

4.2.3 Activation of T cells

Activation of T cells requires binding of the TCR to an antigen loaded MHC molecule. MHC class I is found on all nucleated cells and presents endogenous (e.g. viral components) peptides to CD8 T cells, whereas MHC class II molecules are expressed by professional APC and present peptides via the exogenous pathway to CD4 T cells [4]. However, complete stimulation of a T cell requires additional costimulatory signals. Interactions between accessory molecules on T cells and their ligands on APC enhance the binding between TCR and MHC, which on its own is relatively weak. The co-receptors CD4 and CD8 are expressed on T cells and bind directly to the MHC [23]. This is important to stabilise the binding of MHC to TCR, whereas other molecules provide costimulatory signals and promote, or regulate, the downstream intracellular signalling. An important costimulatory signal is provided by the pairing of CD28 on the T cell and its ligand B7 on APC [24]. Signalling through CD28 and the synthesis of interleukin-(IL)-2 contributes to survival and proliferation of T cells. Another costimulatory signal is provided by the pairing of CD70 on the B cell surface and CD27, which is upregulated following T cell stimulation. This enhances T cell activation, proliferation and differentiation [25, 26]. CD40-ligand (CD40L) is only transiently expressed on CD4 T cells after antigen contact and binds to CD40 on B cells contributing to APC activation and primary T cell responses [27]. These interactions (summarised in Figure 4) result in intracellular complex signalling cascade and activate multiple transcription factors that initiate gene transcription necessary for proliferation and effector cell functions (rev. in [28]).

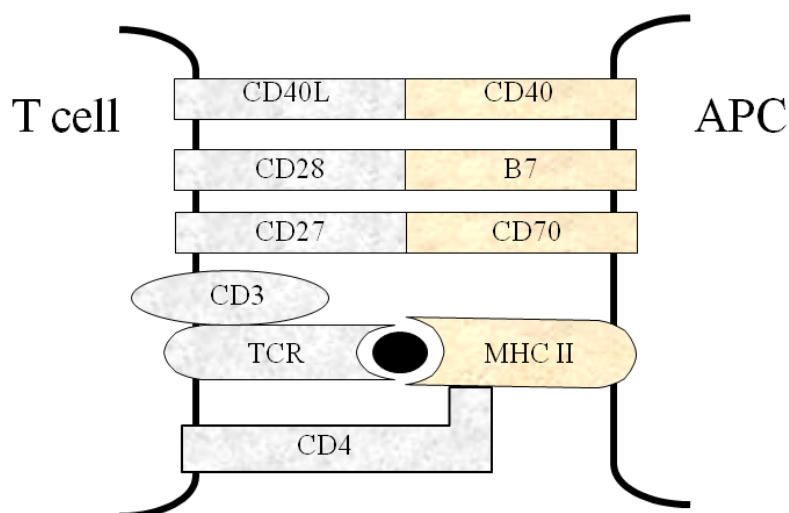


Figure 4: **Receptor-mediated control of T cell activation.** Shown are some receptors on CD4 T cells and APC that play an important role in antigen-specific T cell activation.

4.2.4 Effector mechanisms of T cells

Although CD4 and CD8 T cells are distinct T cell subsets, they possess overlapping functions that can be grouped into early, intermediate and late functions [29]. Calcium flux and phosphorylation of signalling proteins are induced immediately after activation and are therefore early functions, while intermediate functions such as cytokine production and degranulation occur a few hours after stimulation. Late functions include proliferation and apoptosis and can only be detected hours to days after stimulation [29]. In this work, intermediate functions were investigated.

Cytokines are small molecules (<30 kD) secreted by cells of the immune system which carry signals between cells. These immunomodulating proteins, peptides and glycoproteins include interleukins, interferons and chemokines. Each cytokine reacts with a receptor on the surface of the same or another cell. Binding of the cytokine to the receptor triggers a cascade of intracellular signalling which leads to the up and/or downregulation of several genes and their transcription factors. This can result in proliferation, production or inhibition of other cytokines, surface receptors or even suppression of their own effect. The cytokine network is very complex and includes several positive and negative feedback loops, antagonistic and agonistic relationships, pleiotropy and redundancy. Half-lives of cytokines are generally very short which means that new transcriptions and translations are needed for each new stimulus. All these mechanisms help maintain the delicate balance of cytokines to orchestrate the immune response against pathogens without harming the body [1].

In this work, the following activation markers were analysed in detail: IFN γ , TNF, IL2, CD40L and CD107a. IFN γ has antiviral and antiproliferative activities and influences other immune cells. It binds to the IFN γ receptor which is expressed on all cells except erythrocytes. IFN γ can induce the activation of macrophages, upregulate the expression of surface molecules (e.g. MHC class I, MHC class II and B7), influence endogenous antigen processing and promote or inhibit apoptosis. IFN γ is mainly produced by activated CD4 T cells but also by CD8 T cells and NK cells [1]. TNF is a pleiotropic cytokine which has inflammatory, toxic and immunoregulatory effects. TNF is involved in systemic inflammation but its most important effect is the regulation of immune cells. TNF is produced by many cell types upon different stimuli and in various physiological and pathological conditions [1]. T cells which are activated by antigen-binding and CD28 costimulatory signals are the main source of IL2. IL2 is secreted following autocrine signalling and functions as a T cell chemoattractant. It stimulates the proliferation of activated

leucocytes and promotes the differentiation of CD8 T cells and B cells. It is also important for the induction of self-tolerance and homeostasis. [1]. CD40L, also known as CD154, is primarily expressed as a costimulatory surface molecule on activated CD4 T cells. It can also be found on B cells, NK cells, macrophages, mast cells, non-haematopoietic cells as well as in a soluble form. CD40L binds to CD40 on APC and thereby triggers their activation in association with TCR stimulation by MHC molecules. CD40L is transiently upregulated in activated CD4 T cells and can therefore be used as a marker for antigen-specific stimulated CD4 T cells [30, 31]. CD107a and b, also known as lysosomal-associated membrane protein-1 and 2, are proteins located in the cytotoxic granule membrane which become transiently expressed on the T cell surface upon granule exocytosis. Therefore CD107a can be used as a marker for degranulation [32, 33].

4.2.4.1 Detection of activated T cells by flow cytometry

Currently several tools are available for the detection of antigen-specific T cells by flow cytometry. To date, flow cytometry is the most sophisticated method available to analyse T cells with the possibility to simultaneously measure several read-outs on the same cell. Flow cytometric methods include intracellular cytokine staining (ICS), MHC-class-I tetramers and dextramers, measurement of proliferation by CFSE or PKH dyes, PhosFlow and degranulation assays [33-39]. These assays have sufficient sensitivity to measure rare cell populations which is necessary if antigen-specific T cell responses are investigated.

The measurement of intracellular cytokines in T cells after stimulation by flow cytometry was first done by Waldrop *et al.* (1997) to analyse CD4 T cell responses to Cytomegalovirus (CMV) lysate [40]. ICS measured by flow cytometry allows multiparametric analysis on a single cell level and relies on short term *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) (Figure 5). Viral lysates, whole proteins, single peptides and peptide pools spanning whole proteins can be used for antigen-specific stimulation of T cells *in vitro*. Lysates and proteins have to be internally processed by APC before being presented on MHC class I or MHC class II molecules, whereas peptides can be directly taken up by MHC class I or MHC class II molecules and presented to the T cells. Peptides with a length of 8-10 amino acids (aa) are optimised to stimulate CD8 T cells via MHC class I and longer peptides (15-30 aa) are optimised to stimulate CD4 T cells. In addition, T cells can be polyclonally stimulated by superantigens, antibodies or mitogens. In this study, pools of overlapping peptides spanning whole proteins were used. They cover all potential epitopes in the proteins and stimulate both CD4 and CD8 T cells [41, 42].

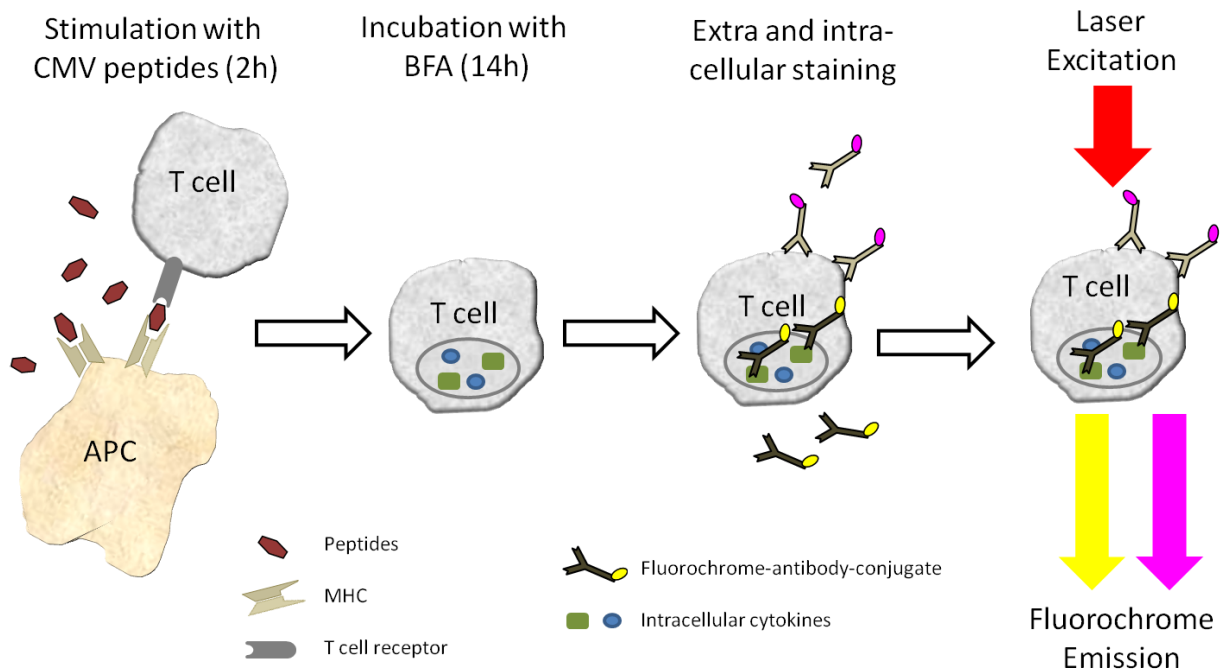


Figure 5: **Overview of flow cytometric procedure.** PBMC were incubated with peptide pools spanning whole CMV proteins. After 2 h BFA was added for 14 h. Subsequently, cells were labelled extra and intracellular with fluorochrome-antibody-conjugates and acquired by using a LSRII flow cytometer.

Produced cytokines are usually secreted from cells. Therefore, to measure what a specific cell is producing golgi stop inhibitors (Monensin or Brefeldin A (BFA)) are added during the stimulation. These compounds stop the secretion of molecules. The subsequent use of permeabilising solutions allows the labelling of cytokines with fluorochrome-conjugated antibodies (Ab) inside the cell.

The improvements of flow cytometry and the development of more fluorochromes give the opportunity to study up to 18 markers on one cell [43]. The number of distinct functional cell subsets increases geometrically with the number of functions measured [44]. Nevertheless, the increasing number of reagents in multicolour flow cytometry also has disadvantages. Unwanted spectral overlaps and measurement errors increase as more fluorochromes are used to label cell markers simultaneously [45]. The use of more colours requires a careful establishment of the entire panel to enable correct analysis of the immune response.

4.3 Cytomegalovirus (CMV)

CMV is a member of the human herpesvirus family which includes the herpes simplex virus, Epstein-Barr virus (EBV), varicella-zoster virus and human herpesvirus 5-8. Herpesviruses are usually characterised by an asymptomatic acute infection followed by lifelong persistence in the human host. Human CMV is highly prevalent in most populations (50-90% seropositivity in developed countries and almost 100% in developing countries). During active infection, the virus is excreted in body fluids, such as urine, saliva, breast milk and semen (rev. in [46]). Infection usually occurs in early childhood from both vertical and horizontal transmission or in young adults by sexual transmission [47]. The infection rate in adults is about 1% per year [48]. Although CMV is usually acquired as an asymptomatic infection at young age, if infection occurs before birth, the consequences can be severe. Congenital CMV is the most common infectious form of congenital birth defects. The prevalence is 0.15-2% worldwide and 10-20% of babies with congenital CMV infection will exhibit neurological damage (rev. in [49]).

An active CMV infection is defined by detectable viral replication or a significant rise in CMV-specific antibodies. Active infection can be a primary infection in a previously seronegative individual or a reactivation in a seropositive individual. If there is no active replication of the virus in the host the infection is latent. CMV disease is the clinical expression of active infection [50].

CMV infects mainly epithelial and endothelial cells, fibroblasts and leucocytes. In the case of CMV disease, the virus can be found in all organs (rev. in [46]). CMV replicates very slowly in cell culture and *in vivo*. The life cycle of CMV can be divided into three phases: immediate early (IE), early, and late. The IE genes are transcribed after virus entry and rely on host factors for expression. Following the IE stage, the early gene products are transcribed and include DNA replication proteins and some structural proteins. Finally, the late gene products are transcribed. These are mainly structural proteins that are involved in virion assembly and shedding of the virus. Formation of the capsids occurs in the nucleus of the host cell and accumulation of these particles results in the typical “owl’s eye” appearance [51].

4.3.1 CMV-specific immune response

CMV is a large icosahedral beta-herpesvirus with a 230 kb double-stranded DNA genome with 213 known open reading frames, including 151 open reading frames whose protein products are

immunogenic for CD4 and/or CD8 T cells [52]. The immune response is not directed evenly against all CMV proteins but is clearly focused. Two of the most immunogenic proteins are the phosphoprotein 65 (pp65) and the IE protein 1 (IE1) [52-58]. The structural pp65 accumulates in both nucleus and cytoplasm of infected cells as the virus matures late in infection while IE1 is a non-structural protein, which is expressed as one of the first CMV-antigens in an infected cell.

Healthy immunocompetent individuals produce anti-CMV immunoglobulin (Ig) M during primary infection, which persists for 3-4 months (rev. in [46]). IgG are produced a few weeks later and persist for life. It has been shown that in primary asymptomatic CMV infection, CMV-specific CD4 T cells appear 10 days after CMV DNA is detectable. This is followed, 14 days later, by the appearance of CMV-specific CD8 T cells [59]. T cell responses to CMV proteins are both advantageous and harmful for the human body. T cell responses are protective against CMV disease but these responses also result in massive clonal expansion of CD8 T cells which compromise host immunity to other infections (rev. in [60]). Reactivation of the latent virus is rare in immunocompetent people and appears to be linked to immune activation and stress. Examples include astronauts during space flights [61, 62], patients in intensive care units [63], after septic shock [64] and patients with Guillain-Barré syndrome [65]. In healthy people, the CD4 and CD8 T cell responses are sufficient to control, but not eradicate the virus.

Millions of years of co-evolution between CMV and humans have enabled the virus to develop mechanisms to modulate the human immune system to survive in the host. Although the host launches a strong immune response against it, CMV is able to establish lifelong persistence. CMV encodes proteins that can influence both the innate and the adaptive immune responses. The proteins include cytokine homologues, chemokine receptor homologues and chemokine binding proteins. CMV can limit the complement cascade, lessen the effects of IFN activation, interfere with NK cell activity, induce anti-inflammatory responses with an IL10 homologue and prevent apoptosis. CMV can also impair T cell activation by dampening the release of IFN γ and TNF from PBMC and downregulating the expression of MHC class I and II (rev. in [66-68]). All of these CMV-mediated modulations result in nonspecific inhibition of cellular and humoral immune responses. This suggests that the virus may employ mechanisms that make the host susceptible to other types of infection. The crucial role of the immune system to control CMV infection can be observed in immunocompromised individuals in which CMV infection or reactivation causes serious and often life-threatening complications, particularly in transplant recipients and patients with acquired immune deficiency syndrome (AIDS). CMV disease is one

of the most frequent opportunistic infections in patients with advanced human immunodeficiency virus (HIV) infection, of whom 40% develop life threatening CMV disease (rev. in [69]). Additionally, in HIV-co-infected individuals, CMV appears to be related to enhanced progression of AIDS (rev. in [70]). Another group with a high risk of severe CMV disease are recipients of transplants [71, 72]. Immunosuppressive treatment following solid organ transplantation weakens the immune response to CMV. As a result high numbers of patients are at risk of CMV associated diseases which can be lethal. More than 50% of transplant recipients show evidence of CMV replication in the first year post-transplantation [73]. CMV infection is therefore the most common opportunistic infection after solid organ transplantation and causes both direct and indirect morbidity [74, 75]. Direct effects are caused by the virus and include nephritis, hepatitis, colitis and myocarditis. Indirect effects are caused by the interaction between virus and host immune system and are associated with an increased risk of graft rejection, opportunistic infections and decreased recipient survival [72, 76].

4.3.2 CMV and Immunosenescence

The terms ‘immune-ageing’ or ‘immunosenescence’ describe a combination of features affecting the functions and phenotypes of immune cells in older age. Older people will have survived numerous infections and therefore have an increased number of memory cells which should enable them attack a previously encountered antigen more effectively. Unfortunately, rather than the immune system becoming more effective with age, at some stage it starts becoming less effective. By comparison with the younger population, many diseases in the elderly are more frequent and more severe. Older individuals are more likely to suffer from vaccine failure and infections caused by both latent carried and newly encountered pathogens, including urinary tract infections, respiratory infections, skin infections, varicella-zoster reactivation and tuberculosis (rev. in [77-79]). Immunosenescence is multi-factorial and so far not completely understood. Normal ageing is determined genetically but external factors also affect ageing in general and immunosenescence in particular. Published research in this field suggests that latent viral (e.g. CMV) infections also contribute to immunosenescence [80-86].

The innate and adaptive immune responses are both affected by the ageing process but the most profound alterations are described in the adaptive response, specifically in T cell immunity (Table 1, rev. in [87-90]). The differences in T cell immunity attributed to ageing include changes on the single cell level as well as changes in T cell populations. The diversity of the TCR is reduced in old people which might explain the impaired T cell immunity to new

infections [91]. The declining output of T cells by the thymus also contributes to immunosenescence. Although T cells are produced continuously throughout life, in elderly people the degradation of the thymus causes a sharp decrease in the number and type of T_N cells produced. Additionally the capacity for renewal of haematopoietic stem cells declines with age. Repeated exposure to antigen induces more differentiated T cells and accelerates T cell ageing. It appears that chronic infections (e.g. CMV, EBV) play an important role because T cells are constantly reactivated [92, 93]. Taken together, this results in an altered memory T cell subset distribution with less T_N cells and more differentiated T cells with a reduced TCR diversity in older people [94, 95]. While the adaptive immune system weakens with age, at the same time an elevated inflammatory response and a chronic systemic pro-inflammatory state, called inflamm-ageing, can be observed [96]. The parallel deterioration of T cell immunity and the upregulation of the innate immunity might be explained by recent reports suggesting that T cells are necessary to regulate the innate immune response (rev. in [97]).

Innate immunity	Adaptive immunity
<ul style="list-style-type: none"> – Increased inflammatory background (Inflamm-ageing) – Impaired antigen-uptake and presentation by APC – Impaired macrophage functions – Reduced NK cell cytotoxicity 	<ul style="list-style-type: none"> – Decreased naive T cell output by thymus – Accumulation of effector and memory T cells – Restricted T cell repertoire – Reduced B cell production – Impaired B cell functions – Decreased CD4/CD8 T cell ratio

Table 1: Age-related changes of the immune system

Swedish longitudinal studies in elderly individuals have shown that immunological parameters can be used to predict mortality [98, 99]. Low CD4/CD8 ratio, poor proliferative response and high numbers of CD28⁻ T cells are associated with CMV and contribute to the immune risk profile (IRP) which is highly associated with mortality in older people. Although studies did not show a direct correlation between CMV infection and mortality, the results suggest a contribution of the virus to risk factors that correlate with mortality (rev. in [80, 84]). It is still unclear to what extent persistent viral infection in general and CMV in particular contribute to immunosenescence.

To gain more information about the complex interaction of the ageing immune system and CMV infection, experiments were performed on PBMC from CMV-seropositive and CMV-seronegative people in three different age groups. Phenotypes and functionality of antigen-specific and polyclonally stimulated PBMC were investigated by multicolour flow cytometry. The expression of the phenotypic markers CD45RA and CD27 and activation markers CD40L, IFN γ , IL2 and TNF were analysed on CD4 and CD8 T cells.

Furthermore, the *in vitro* effect of immunosuppressive drugs on polyfunctional CMV-specific T cells was analysed to expand the understanding of CMV reactivation in transplant patients. For this purpose, PBMC from CMV-seropositive donors were incubated overnight with immunosuppressive drugs and subsequently stimulated with pp65. In addition to the aforementioned activation markers, expression of CD107 as a marker of degranulation was also analysed.

5 Materials and Methods

5.1 Materials

5.1.1 Reagents

Dimethyl sulfoxide (DMSO)	Pierce, Rockford, USA
Ficoll-Paque TM PLUS	Biochrom AG, Berlin, Germany
Trypan blue	Biochrom AG, Berlin, Germany
Acetic acid	Merck, Darmstadt, Germany
Bovine Serum albumine (BSA)	Serva, Heidelberg, Germany
Natriumazid (NaNH ₃)	Serva, Heidelberg, Germany
Brefeldin A	Sigma, USA
GolgiStop	BD, San Jose, CA, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma, USA
FACSFlow	BD, San Jose, CA, USA
FACS Lysing Solution	BD, San Jose, CA, USA
FACS Permeabilizing Solution	BD, San Jose, CA, USA
Fetal calf serum (FCS)	Biochrom AG, Berlin, Germany
Phosphate buffered saline (PBS)	PAA, Pasching, Austria
Paraformaldehyde (PFA)	Biochrom AG, Berlin, Germany
Penicillin	Biochrom AG, Berlin, Germany
Streptomycin	Biochrom AG, Berlin, Germany
L-Glutamine	Biochrom AG, Berlin, Germany
Cyclosporine A (CSA)	Sigma, USA
Tacrolimus (FK506)	Sigma, USA
Rainbow Beads	BD, San Jose, CA, USA
Cytometer Setup Tracking (CST) Beads	BD, San Jose, CA, USA
CompBeads	BD, San Jose, CA, USA

5.1.2 Media and buffer

RPMI 1640 Medium	Biochrom AG, Berlin, Germany
Complete media	RPMI 1640 Medium, 100 U/ml Penicillin, 100 U/ml Streptomycin, 2 mM L-Glutamin, 10% FCS
Fixation buffer	0.5% PFA in PBS

5.1.7 Antibody panels

Laser	Excitation	Emission	Fluorochrome	Antigen	Clone	Amount	Distributor
blue	480	613	ECD	CD4	SFC112T4D11	2 µl	Beckman Coulter
	490	675	PerCP	CD45	2D1	20 µl	BD
	696	719	APC-H7	CD8	SK1	3 µl	BD
violet	403	455	PB	CD3	OKT3	1 µl	Biolegend

Table 2: Staining reagents for the whole blood staining

Laser	Excitation	Emission	Fluorochrome	Antigen	Clone	Amount	Distributor
blue	494	520	FITC	CD16	3G8	5 µl	BD
	480	578	PE	CD27	L128	5 µl	BD
	480	613	ECD	CD45RA	MB1	5 µl	Beckman Coulter
	490	675	PerCP	CD4	SK3	15 µl	BD
	480	767	PECy7	IFN γ	B27	4 µl	BD
red	650	660	APC	IL2	5344.111	5 µl	BD
	650	767	Alexa700	TNF α	Mab11	3 µl	BD
	696	719	APC-H7	CD8	SK1	3 µl	BD
violet	403	455	PB	CD40L	24-31	0.5 µl	Biolegend
	367	526	Aqvid			0.75 µl	Invitrogen
	405	605	Qdot	CD3	OKT3	1 µl	Invitrogen

Table 3: Staining reagents for the CMV study

Laser	Excitation	Emission	Fluorochrome	Antigen	Clone	Amount	Distributor
blue	494	520	FITC	CD107a	H4A3	10 µl	BD
	480	578	PE	CD40L	TRAP1	10 µl	BD
	480	613	ECD	CD4	SFC112T4D11	2 µl	Beckman Coulter
red	650	660	APC	IL2	5344.111	5 µl	BD
	650	767	Alexa700	TNF α	MAB11	3 µl	BD
	696	719	APCH7	CD8	SK1	3 µl	BD
violet	403	455	PB	CD3	OKT3	1 µl	CambridgeBioscience
	367	526	Aqvid			0.75 µl	Invitrogen

Table 4: Staining reagents for the experiments with immunosuppressants

5.2 Methods

5.2.1 PBMC Preparation

Heparinised blood was diluted with sterile PBS (1:1) and carefully layered onto Ficoll. Tubes were centrifuged (20 min, room temperature (RT), 1000 x g, no brakes, low acceleration) and PBMC were aspirated and washed twice with 50 ml sterile PBS (10 min, RT, 300 x g). PBMC were diluted in complete medium to a final concentration of 5×10^6 PBMC/ml.

5.2.2 Qdot-Conjugation OKT3

CD3 antibodies (Ab) conjugated to QDot605 were not available at the beginning of the study therefore CD3 (OKT3) Ab were conjugated with the help of a QDot conjugation Kit (Invitrogen) according to the manufacturer's instructions. All steps were done at RT. Briefly, QDots were activated with 1 mM succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate for 1 h while 300 μ l Ab were reduced with 20 mM dithiothreitol for 30 min. QDots and Ab were filtered separately by using NAP-5-columns, 500 μ l of each were collected, mixed and conjugated for 1 h. The conjugation was quenched by adding β -Mercaptoethanol for 30 min at RT. The QDot-Ab-conjugate was concentrated using ultrafiltration devices and separated from the unconjugated Ab with a separation column.

5.2.3 Whole Blood Count

Heparinised whole blood was diluted 1:20 in 3% acetic acid and white blood cells were counted using a haemocytometer to enable the calculation of absolute cell numbers per ml blood.

5.2.4 Whole Blood Staining Procedure

Whole blood (100 μ l) was incubated with Ab specific for surface markers (30 min, 4°C). Red blood cells were lysed with 1 ml BD Lyse (10 min, RT). Following lysis cells were washed with 3 ml washing buffer and acquired immediately.

5.2.5 Stimulation

PBMC (2×10^6) were incubated for 2 h (37°C, 5% CO₂) in 0.5 ml complete RPMI media and either 1 μ g/ml pp65 peptide pool, 1 μ g/ml IE-1 peptide pool, 10 μ g/ml PPD, 0.5 μ g/ml OKT3 as positive control or an equivalent amount of DMSO as negative control. After 2 hours of

incubation, Brefeldin A (10 µg/ml) was added and the volume was adjusted to 1 ml (complete media). Cells were then incubated for 14 h (37°C, 5% CO₂).

5.2.6 Incubation with immunosuppressants

PBMC (2×10^6) were incubated overnight (37°C, 5% CO₂) in 1 ml complete media in the presence of FK506 (2 ng/ml, 10 ng/ml or 50 ng/ml), CSA (40 ng/ml, 200 ng/ml or 1000 ng/ml) or without immunosuppressant. After 16 hours cells were washed with complete RPMI media and resuspended in 500 µl of complete RPMI media with 10 µl CD107a-FITC, 1 µl Golgistop and 1 µg/ml pp65 spanning peptide pool or an equivalent amount of DMSO. After 2 hours of incubation 2 µl Brefeldin A (5 mg/ml working solution in DMSO) were added and the volume adjusted to 1 ml (complete media) for the last 4 hours.

5.2.7 Immunofluorescence Staining Procedure

Following stimulation, cells were incubated with 20 mM EDTA in PBS. Cells were washed using wash buffer and incubated with pre-titered surface antibodies for 30 min at 4°C in the dark. Cells were washed, lysed with BD Lyse and permeabilised with BD Perm2 solution according to the manufacturer's instructions (BD Bioscience). Following permeabilisation cells were stained intracellularly for 30 min at 4°C in the dark with pre-titered antibodies. Cells were washed, fixed in fixation buffer and analysed by flow cytometry.

5.2.8 Flow Cytometric Analysis

FlowJo 8.8.6 software was used for data analysis. Lymphocytes were gated in a side scatter (SSC) vs. forward scatter (FSC) gate and doublets were excluded using FSC-height vs. FSC-area. Live cells were gated using aqua viability dye (aqvid) and NK cells were excluded by gating out bright CD16/CD56 cells. CD3⁻ cells were excluded by gating CD3 against CD4 and CD8 cells were excluded by gating CD4 against CD8. To take into account the downregulation of CD4 on activated cells, CD4 T cells were gated against each activation marker and then combined into one CD4 T cell population. The same strategy was applied for CD8 T cells. CD4 and CD8 T cells were analysed separately regarding their CD45RA and CD27 expression as well as their expression of the four activation markers (CD40L, IL2, TNF, IFNγ) in all possible combinations. The four subsets CD45RA⁺CD27⁺, CD45RA⁻CD27⁺, CD45RA⁻CD27⁻ and CD45RA⁺CD27⁻ were also analysed in respect to their activation markers. The four activation markers were gated against CD4 or CD8. The gating strategy is shown in Figure 6 and Figure 7.

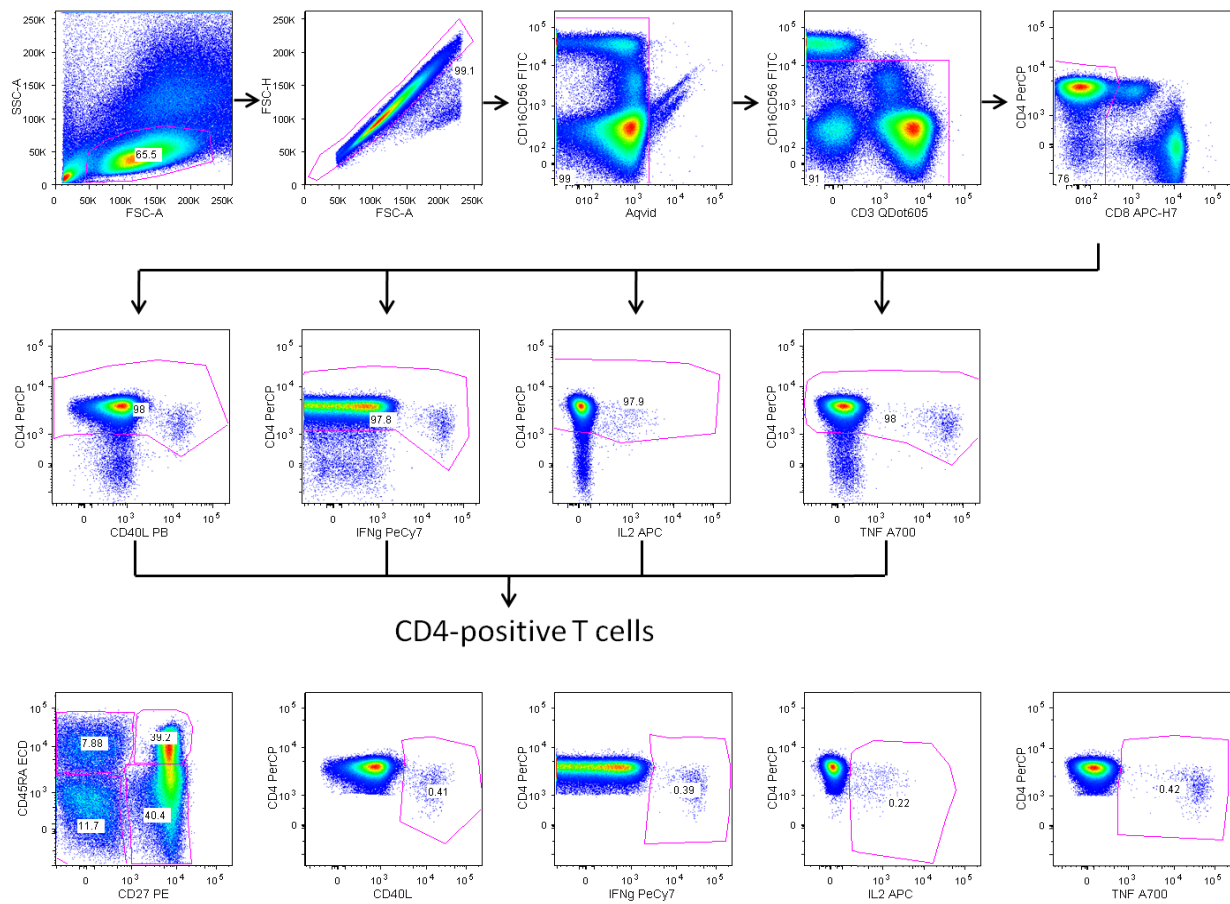


Figure 6: Gating Strategy Part 1: Lymphocytes were gated in a SSC vs. FSC gate, doublets were excluded using FSC-height vs. FSC-area. Live cells were gated using aqvid and NK cells were excluded by gating out CD16-positive cells. CD3⁻ cells were excluded by gating CD3 against CD4. CD4 and CD8 T cells were gated separately in a CD4 against CD8 plot. CD4 T cells were gated against each activation marker and then combined into one CD4 T cell population. CD4 were analysed regarding their CD45RA and CD27 expression as well as their expression of the four activation markers (CD40L, IFN γ , IL2, TNF). Shown are plots of pp65 stimulated PBMC from one representative middle-aged CMV-seropositive donor.

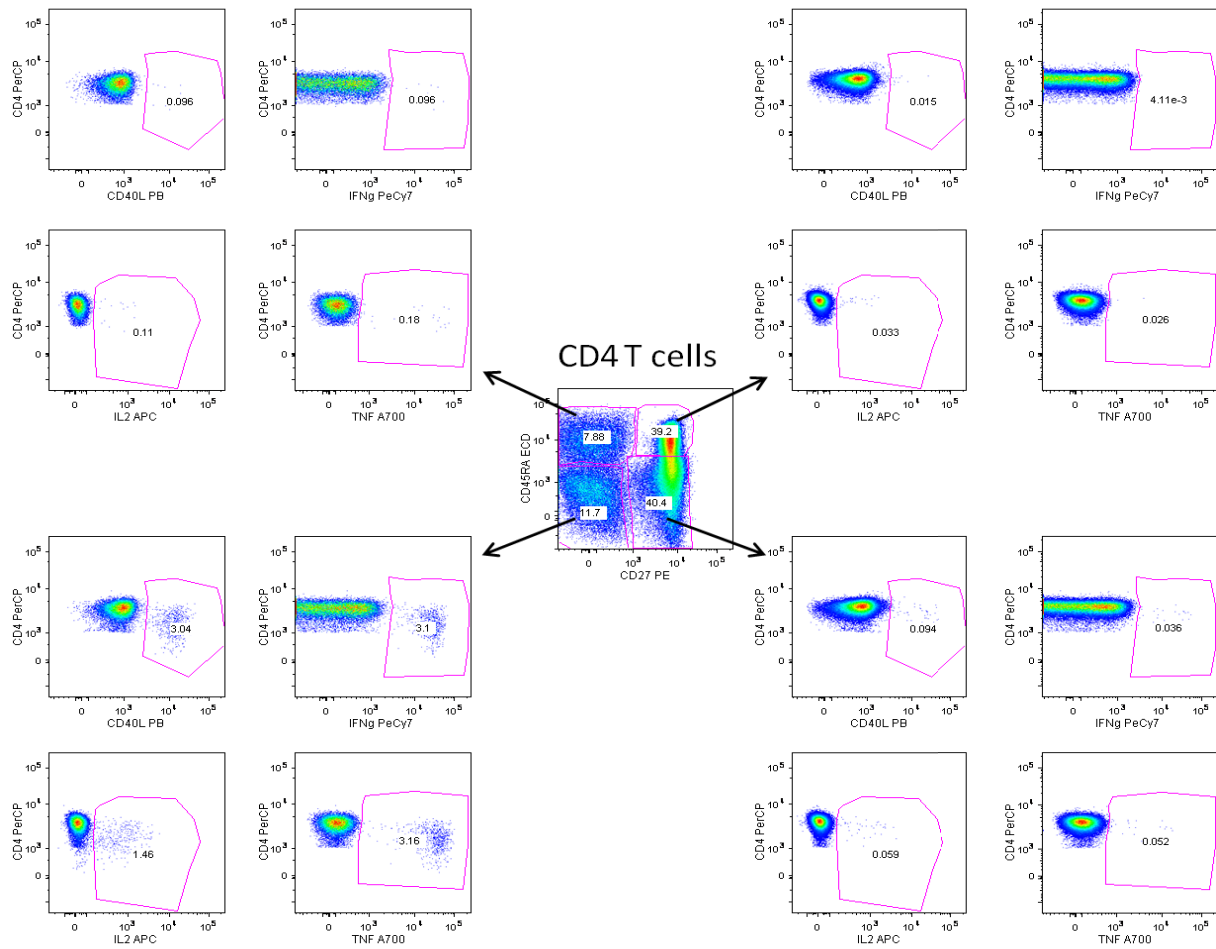


Figure 7: **Gating Strategy Part 2:** Each of the four memory CD4 T cell subsets was analysed for intracellular expression of the for activation markers CD40L, IFN γ , IL2 and TNF. Shown are plots of pp65 stimulated PBMC from one representative middle-aged CMV-seropositive donor.

The different functional subsets were generated as a combination of all four markers totalling 15 subsets (n^4 excluding the quadruple negative subset) by boolean gating (Figure 8). Boolean gates represent all combinations (plus and minus) of a set of gates and can be automatically created by FlowJo. For example, if a sample was stained for IFN γ and TNF, ‘boolean combination’ creates the four (n^2) gates that represent all combinations of expression of the markers (e.g., IFN γ^+ TNF $^+$, IFN γ^+ TNF $^-$, IFN γ^- TNF $^+$ and IFN γ^- TNF $^-$).

The frequency of cells expressing a certain marker was calculated in relation to the number of cells in the parental subset. Unstimulated samples were used as negative controls and subtracted from stimulated samples to control for background activation in each subset. All presented data are background subtracted.

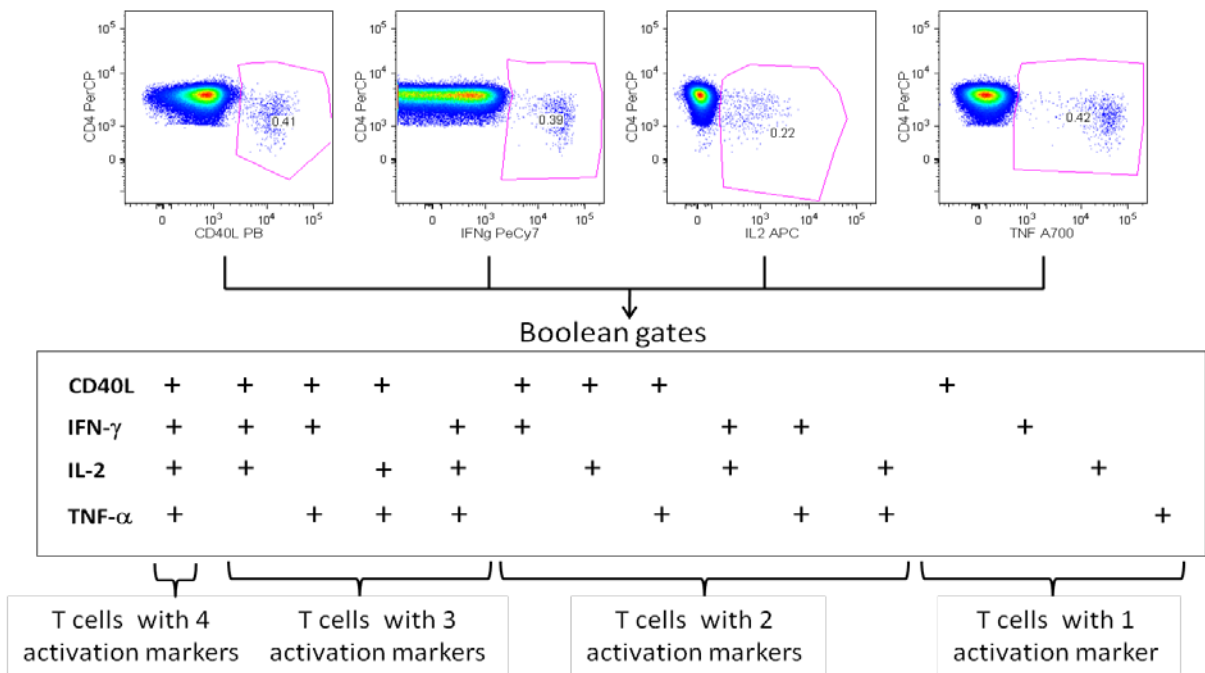


Figure 8: **Boolean gating:** Activation marker co-expression profiles were determined using the boolean gating function of FlowJo software.

5.2.9 Statistical Analysis

Statistical analysis was performed using SPSS 16. Normal distribution was tested using the Kolmogorov–Smirnov test and approximately 80% of the data were determined to be non-normally distributed. Therefore, data are displayed as median and range. In cases where the frequency of all responding T cells is used as a reference population, stacked bar diagrams display the mean to total 100% graphically and allow a direct comparison.

For consistency, all statistical tests were carried out for non-normally distributed data. The resulting p-values for non-parametric data can also be applied for parametric data without loss of significance. Correlation was carried out using Spearman’s correlation coefficient R_s . Mann-Whitney-Test was used to test for significance between groups and significance between distributions was tested by multivariate analysis for covariance. P -values less than 0.05 were considered significant. Mean fluorescence intensity (MFI) can differ between different experiments and therefore had to be standardised to allow comparisons between different subjects. Individual values were standardised by subtracting the population mean from the individual value and then dividing the difference by the population standard deviation. These standardised MFI values are displayed as line curves, showing the median and 95% confidence

interval (CI). In scatter plots, each dot represents an individual donor and a linear regression line is shown. Box plots display the median, interquartile range (IQR, difference between the 75th and 25th percentiles), minimum, maximum, outlier (•, more than 1.5 IQR away from 75th and 25th percentiles) and extreme values (*, more than 3 IQR away from 75th and 25th percentiles).

Improvements in flow cytometric methods, especially polychromatic intracellular flow cytometry, enable researchers to generate millions of data points simultaneously for individual samples. In this study the investigation of four different activation markers at the same time resulted in 16 exclusive combinations of activation markers. One population consisted of quadruple negative cells which were considered to be non-responding cells. The other 15 populations included all combinations from single cytokine producers to cells producing all four activation markers simultaneously. Since multicolour flow cytometry is a relative new technology, there is no consent yet of what statistical methods are best applied. So far, research groups have used a variety of different statistical methods to analyse the data and define significance between different cell populations.

One important question was whether the frequencies of cells or absolute numbers per ml blood should be analysed. This report uses 'absolute counts' for numbers of cells per ml blood and 'frequencies' when indicating the percentage of a subpopulation with respect to the relevant reference population (e.g. IFN γ -producing CD4 T cells in percent of all CD4 T cells). The analysis of polyfunctional cells is very complex and can be done in several ways which hinders direct comparison between data generated in different laboratories. When frequencies are used for the analysis, the results depend entirely on the reference population. The same data could show either an increase in a specific subpopulation related to all T cells or demonstrate a decrease by using all responding T cells as a reference population. If frequencies are used, it is fundamental to write precisely what reference population was used (e.g. all CD8 T cells or all responding T_N CD4 T cells) to interpret the data appropriately. In this work, the frequencies with the reference population indicated were used for graphs. In many cases, for comparison, supplemental graphs displaying absolute numbers per ml blood are shown in the appendix. Analyses were done for both frequencies and absolute numbers per ml blood for the CMV-study. Due to the prolonged *in vitro* incubation with immunosuppressive drugs (which may affect T cell numbers), frequencies but not absolute numbers were investigated in the experiments with the immunosuppressants.

Another important consideration was the large interindividual variations in the same subsets. This is especially important considering that some of these subsets were very small (only consisting of a few cells) and therefore even small variations had a large impact. The precision of the quality of the response is based on the number of cells acquired. The acquisition of higher numbers of PBMC would allow the analyses of more responding cells, but the number of analysed cells could not be expanded since there were only limited amounts of blood available. The cost for reagents would have multiplied if larger amounts of PBMC had been processed and analysed. It was unclear if these very small subsets based on a small number of events were representing real subsets or if they were false positives and represent non-specific staining of cells. These small subsets could be excluded from the data. However, this would lead to the overestimation in the frequencies of the remaining larger subsets and skew the analysis. Subsets which existed in less than half the donors came up with zero in the median and therefore did not influence final statistical analyses. Small subsets that appeared in more than half the donors were likely to be real subsets of cells and were not excluded. Therefore, the only cut-off in this study was performed when the frequencies of all responding CD4 or CD8 T cells were below 0.01% of all CD4 or CD8 T cells after background subtraction. This cut-off did not exclude any responses after OKT3 stimulation in any donor or any pp65-specific responses in CMV-seropositive donors.

The complexity of the T cell response provided a further challenge of the data analyses. The investigation of four different activation markers resulted in 15 exclusive cell subsets expressing different combinations of activation markers. These 15 populations were examined in the four memory subsets and in the total CD4 and CD8 T cell compartment. This resulted in 150 distinct populations of activated T cells. For further analysis these populations were grouped together. This allowed investigation of e.g. all responding T cells in T_{EMRA} cells, all CD4 T cells that produced IFN γ or all CD8 T_N cells that synthesised three cytokines simultaneously. All data were analysed in terms of percentages and as absolute numbers per ml blood. Taken together, 1125 different T cell subsets per sample were examined in this work. Additionally, MFIs were acquired for phenotypic and activation markers and analysed for several T cell subsets. The data were analysed separately for each stimulated sample (DMSO, pp65, IE1, PPD and OKT3) and for each stimulated sample with the background subtracted (pp65-DMSO, IE1-DMSO, PPD-DMSO and OKT3-DMSO) for each of the 50 tested individuals. For this study, more than half a million data were analysed, not including the study with the immunosuppressive reagents.

6 Results

6.1.1 Staining Panel Development

In order to investigate polyfunctional T cell responses an 11-colour reagent panel had to be developed. For all experiments, a LSRII flow cytometer with three lasers (excitation lines at 405nm, 488nm and 635nm), 11 fluorescent detectors and FACSDiva software was used.

Several factors were taken into consideration for the development of the multicolour staining panel [100, 101]. In general, brightest fluorochromes should be used for rare or weakly expressed markers of interest (e.g. cytokines) whereas dim fluorochromes could be used for dump channels to exclude unwanted cell populations (e.g. B cells, NK cells), or for highly expressed, and therefore easily distinguishable, markers (e.g. lineage markers such as CD3, CD4). The functional definition of reagent brightness is the stain index which takes into account the difference between the positive and negative populations and the spread of the negative population. Bright fluorochromes include PE and APC, while APC-H7 and PerCP are dim fluorochromes. It is important to choose the brightest fluorochromes available and to minimise spillover between channels. Spillover negatively affects the cell population, even after compensation is applied, and should therefore be minimised from the beginning.

The first step in the development of the multicolour panel used in this study was the titration of every fluorochrome-Ab conjugate. This was done to detect the best concentration to optimise the signal-to-noise ratio and minimise background staining. Titration was done under assay conditions and at least three different concentrations of each fluorochrome-Ab conjugate were tested. Ab for phenotypic markers were titrated on unstimulated cells whereas Ab for activation markers were titrated on polyclonally stimulated cells and unstimulated cells in parallel. The optimal concentration for each Ab was identified by comparing both background staining and the stain index. For each marker, several different fluorochrome-Ab conjugates were tested and compared with each other. The best possible fluorochrome-Ab conjugate combination was chosen for the panel. High background staining can be caused by dead cells which often bind monoclonal Ab non-specifically. A viability dye was used to exclude dead cells from analyses and improve the performance. Amine-reactive viability dyes penetrate the damaged cell membranes of dead cells, bind to cytoplasmic components and form stable fluorescent aggregates [102, 103]. Finally, the different fluorochrome-Ab conjugates were combined to

check for overspill and blocking of binding due to steric hinderance. To check the sensitivity and overspill of the whole panel, fluorescence-minus-one controls (FMOs) were performed on polyclonally stimulated PBMC (Figure 9). FMOs combine all the reagents of a chosen panel except the one fluorochrome-Ab conjugate of interest and this was carried out for each conjugate. FMOs in Figure 9 show that all phenotype and activation markers are stained specifically and that there is no overspill from other fluorochromes after compensation.

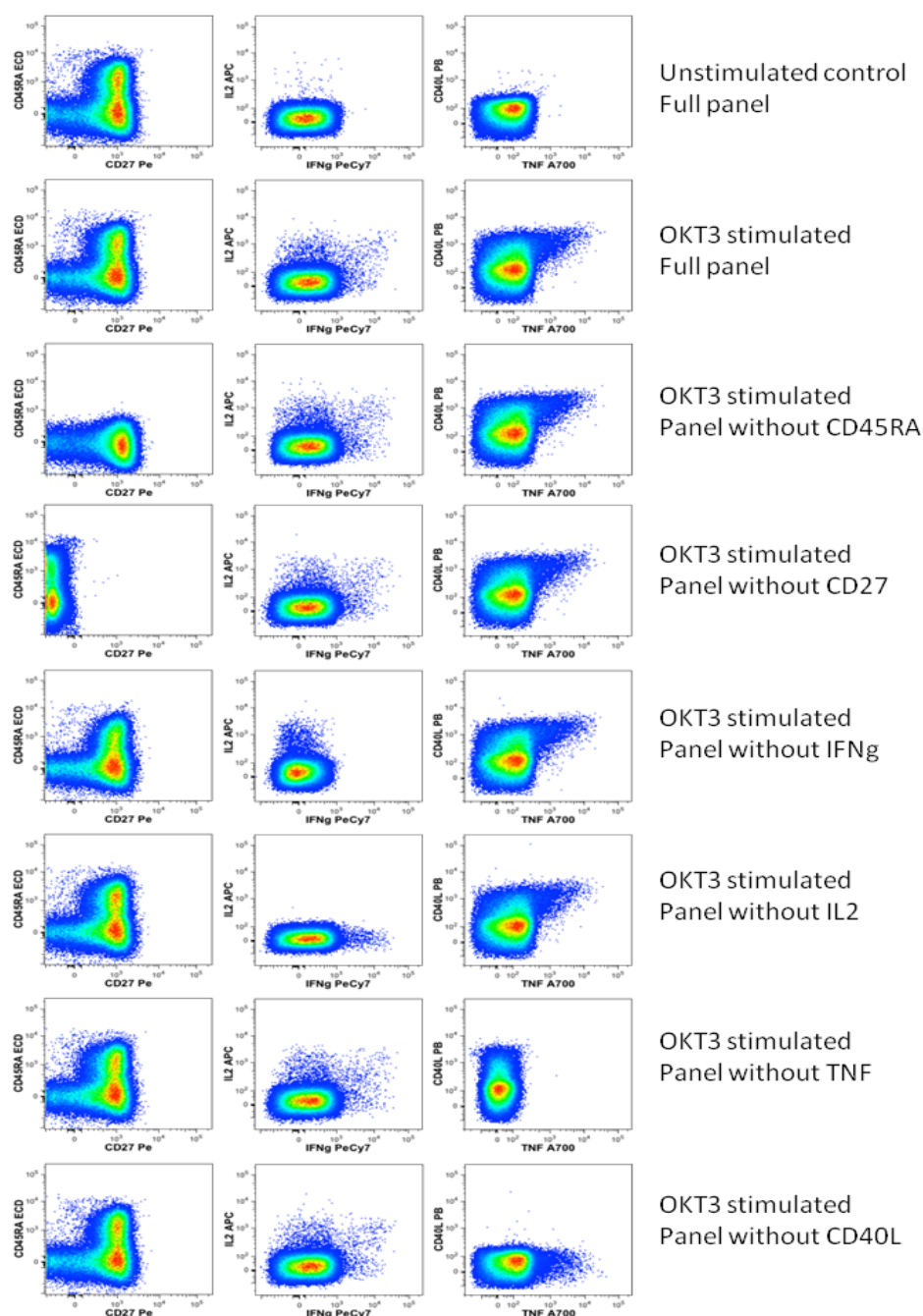


Figure 9: **FMO-controls.** Shown are representative pseudo-colour dot plots from one middle-aged donor. Left plots display CD45RA against CD27, middle plots display IL2 against IFN γ and right plots display CD40L against TNF.

Compensation controls were always run prior to sample acquisition. Commercially available CompBeads were used for the compensation since compensation is specific for the fluorochrome and not the cell type [104]. Adjustment of photomultiplier tube (PMT) voltages was done either manually, after the acquisition of eight-peak-beads, or automatically by ‘Cytometer setup and Tracking’ (CST) following FACSDiva update. The adjustment of PMT voltages was carried out to guarantee a consistent and comparable read-out [105].

Due to practical limitations, such as instrument optical configuration, commercial reagent availability and fluorescence spillover, the following panel was established for the CMV study (Table 5). Aqvid was used to exclude dead cells. CD16 and CD56 were used as a dump channel to exclude NK cells from the analysis. CD3, CD4 and CD8 were used to identify T cell populations. Four different activation markers were analysed to increase sensitivity: CD40L, IFN γ , TNF and IL2. The brightest available fluorochromes were used for the four activation markers to enable gating of activated T cells. The fluorochromes for differentiation markers CD45RA and CD27 were chosen to ensure the best possible separation of the four memory subsets without compensation problems. Dimmer fluorochromes were used for the lineage markers and the dump channel, since these are highly expressed and can therefore be separated with dim fluorochromes.

Fluorochrome	FITC	PE	ECD	PerCP	PECy7	APC	A700	APC-H7	PB	Aqvid	QDot
Antigen	CD16,CD56	CD27	CD45RA	CD4	IFN γ	IL2	TNF α	CD8	CD40L		CD3

Table 5: Staining reagents used in the CMV study

The study involving immunosuppressive drugs was focused on polyfunctional T cells and not on memory differentiation markers, so the panel was altered accordingly (Table 6). CD107a, a degranulation marker, was added to investigate a broader range of effector functions. CD40L-PE was used because this antibody gave better separation and less background staining than the CD40L-PB-conjugate. CD3 and CD4 were used with PB and ECD respectively since these conjugates also gave a better separation.

Fluorochrome	FITC	PE	ECD	PECy7	APC	A700	APCH7	PB	Aqvid
Antigen	CD107a	CD40L	CD4	IFN γ	IL2	TNF	CD8	CD3	

Table 6: Staining reagents used in the experiments with immunosuppressive drugs

6.2 T cells

To investigate age-dependent changes in T cells, PBMC from 26 young, 12 middle-aged and 12 old donors were analysed by flow cytometry for relative cell numbers, phenotypic T cell markers and intracellular cytokine staining (Table 7). Due to technical problems, absolute numbers of cells per ml peripheral blood were only analysed for 24 young, 11 middle-aged and 12 old donors. Leucocytes in the peripheral blood were counted and whole blood staining (CD45, CD3, CD4 and CD8) was performed, which identifies all white blood cells by the expression of CD45 and the T cell subsets as a percentage of all CD45⁺ cells.

Frequencies	n	Median age (range)
Young	26	28 (20-35) years
Middle-aged	12	46 (36-56) years
Old	12	67 (63-84) years
Absolute counts	n	Median age (range)
Young	24	28 (20-35) years
Middle-aged	11	46 (36-56) years
Old	12	67 (63-84) years

Table 7: Number and age distribution of donors

Absolute counts and frequencies of CD3, CD4, CD8 T cells and CD4/CD8 T cell ratios did not differ significantly between the three age groups (Table 8). The absolute numbers of CD4 and CD8 T cells per ml blood and the respective frequencies of these subsets in terms of CD3 T cells were strongly correlated ($R_s > 0.550$, $p < 0.001$ for all tested populations).

	Young 20-35 y	Middle-aged 35-56y	Old 63-84y
Lymphocyte count (10^5 /ml blood) median (range)	62 (32-111)	50 (42-83)	61 (43-86)
% CD3 T cells of lymphocytes median (range)	74 (54-87)	71 (53-76)	73 (44-87)
% CD4 T cells of CD3 ⁺ T cells median (range)	55 (39-71)	63 (43-79)	63 (33-72)
% CD8 T cells of CD3 ⁺ T cells median (range))	34 (24-44)	29 (19-45)	29 (13-59)
CD3 T cell count (10^4 /ml blood) median (range)	140 (78-277)	143 (48-231)	158 (87-225)
CD4 T cell count (10^4 /ml blood) median (range)	73 (45-161)	90 (21-148)	86 (34-130)
CD8 T cell count (10^4 /ml blood) median (range)	44 (22-94)	40 (22-68)	38 (21-131)
Ratio CD4/CD8 median (range)	2.1 (0.6-4.2)	2.4 (1.5-4.6)	2.3 (1.1-5.0)

Table 8: Lymphocyte counts from peripheral blood in three age-groups

As described previously, absolute counts for CD3, CD4 and CD8 T cells were higher in CMV-seropositive than CMV-seronegative donors [106-108]. Only the differences for CD3 and CD8 T cell counts reached statistical significance (Table 9, $p=0.002$ and $p<0.001$ respectively). The frequency of CD8 T cells was also significantly higher in CMV-positive than in CMV-negative donors ($p=0.026$). This resulted in a decreased CD4/CD8 ratio ($p=0.014$) in CMV-infected donors. This result is supported by previous investigations [106, 109]. Of note, only one young donor in this study had a CD4/8 ratio below 1.

	CMV-negative	CMV-positive	p-value
N	25	24	
Lymphocyte count ($10^5/\text{ml}$ blood) median (range)	56 (33-94)	60 (32-111)	ns
% CD3+ T cells of Lymphocytes median (range)	70 (53-85)	73 (44-87)	ns
% CD4+ T cells of CD3+T cells median (range)	58 (39-79)	52 (33-75)	ns
% CD8+ T cells of CD3+T cells median (range)	33 (13-45)	39 (22-58)	0.026
N	24	23	
CD3+ T cell count ($10^4/\text{ml}$ blood) median (range)	121 (48-216)	152 (87-277)	0.002
CD4+ T cell count ($10^4/\text{ml}$ blood) median (range)	73 (21-113)	82 (34-161)	ns
CD8+ T cell count ($10^4/\text{ml}$ blood) median (range)	39 (21-80)	59 (35-131)	<0.001
Ratio CD4/CD8 median (range)	2.4 (1.4-5.0)	1.9 (0.6-4.2)	0.014

Table 9: Lymphocyte counts from peripheral blood in CMV-seronegative and CMV-seropositive donors

6.3 Memory T cell subsets

6.3.1 Influence of age on memory T cell subsets

Memory T cell subsets undergo changes in the course of ageing which are caused by a variety of factors including the decline of thymic output and recurring antigen encounters. To investigate the memory subsets found in peripheral blood in relation to donor age, PBMC were stained for the phenotypic markers CD45RA and CD27 and analysed by flow cytometry. In this work, CD45RA⁺/CD27⁺ cells will be referred to as naïve T cells (T_N cells), CD45RA⁺/CD27⁺ T cells as central memory T cells (T_{CM} cells), CD45RA⁺/CD27⁺ T cells as effector memory T cells (T_{EM} cells) and CD45RA⁺/CD27⁺ cells as effector memory revertant T cells (T_{EMRA} cells) (Figure 10). This is for simplicity and by convention as this nomenclature is used in published reports [14, 110, 111].

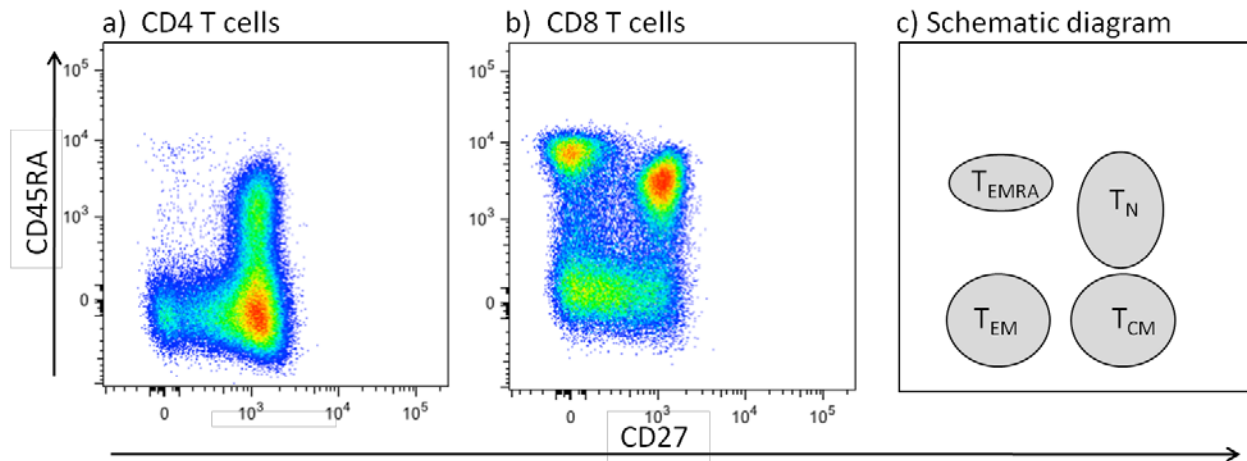


Figure 10: **Memory subsets in CD4 and CD8 T cell compartments.** Representative pseudo-colour dot plots from one middle-aged donor show the distribution of CD45RA and CD27 in a) CD4 T cells and b) CD8 T cells. The schematic distribution of the four memory subsets is represented in c).

In the CD4 T cell subset, the frequencies of T_N cells decreased with age while T_{EM} and T_{EMRA} cells increased, but these weak correlations were not statistically significant (Figure 11). No changes were observed for T_{CM} cells.

In accordance with other publications, the results in the CD8 T cell subset demonstrated that although there is large individual variation, even between people of roughly the same age, the frequencies of T_N cells decreased significantly with age whereas the frequencies of T_{EM} and T_{EMRA} cells increased significantly (Figure 11, $R_s = -0.614$, $p < 0.001$, $R_s = 0.382$, $p < 0.006$ and $R_s = 0.492$, $p < 0.001$ respectively) [112-115]. No changes were observed for CD8 T_{CM} cells. Both CD4 and CD8 T cells underwent the same phenotypic changes during ageing, but the changes were more pronounced in CD8 T cells.

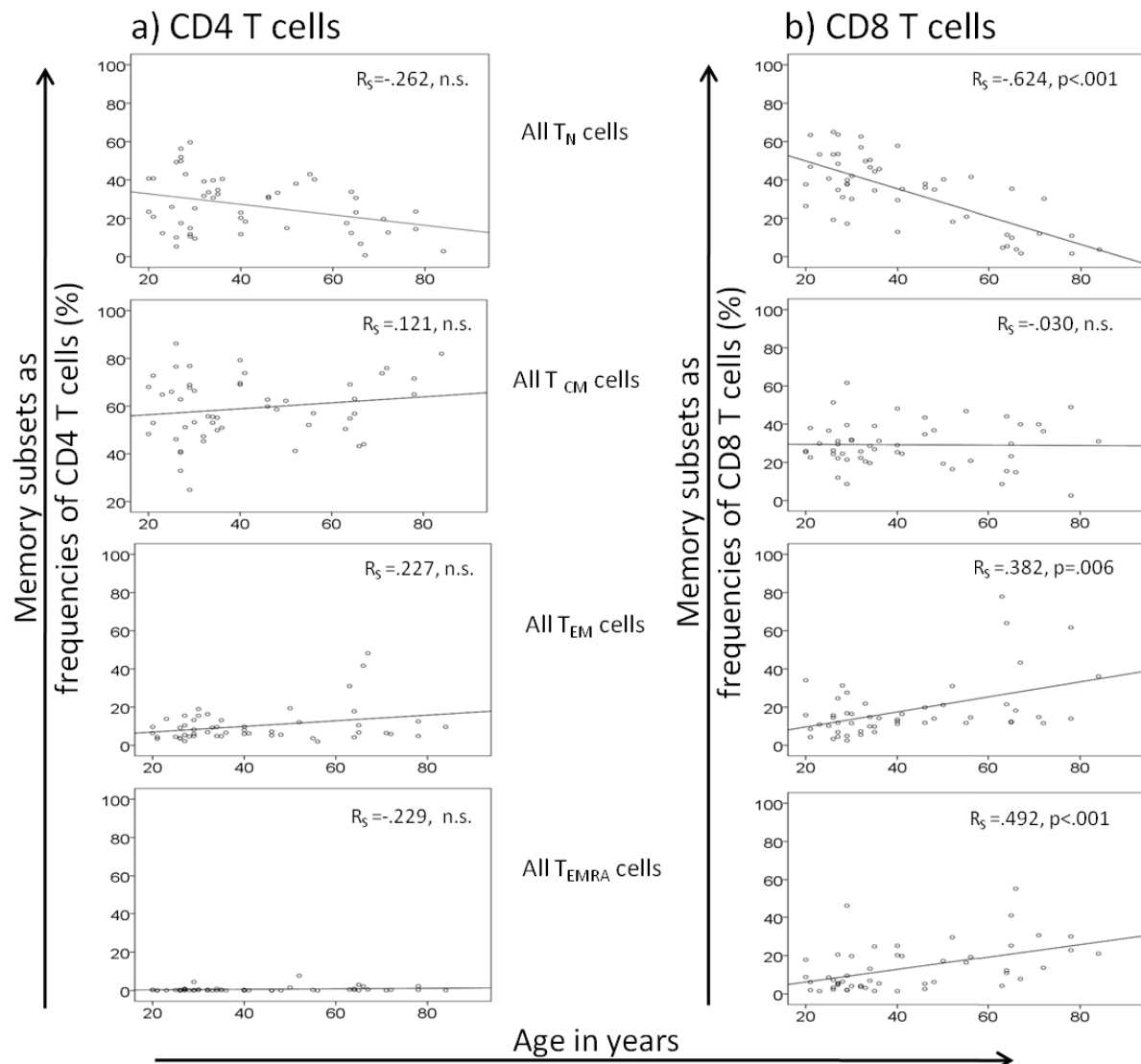


Figure 11: **Correlation of memory T cell subsets and age.** Charts represent the frequencies of all a) CD4 and b) CD8 T cells in T_N , T_{CM} , T_{EM} and T_{EMRA} cells in correlation to age ($n=50$).

To investigate the differences between the four memory T cell subsets, the expression of CD3 and the co-receptors CD4 and CD8 were analysed. T cell activation is known to result in the downregulation of the TCR complex. This study investigated if advanced memory differentiation was also associated with a reduction in TCR complex expression. The four memory subsets were compared for their surface expression of CD3 and CD4 or CD8. For this purpose, the MFI of these molecules was measured on unstimulated T cells. The mean number of surface molecules per cell in a subset correlates with the MFI of the same subset with respect to the fluorescence marker used to detect these molecules. A decrease in MFI corresponds to the loss of surface molecules. While a comparison between measurements using different antibodies cannot easily

be done, comparison between measurements using the same antibody can be made. These changes can be semi-quantified as long as the measurement is made within the linear range of the instrument. In the linear range a proportional increase in fluorescence corresponds to the same proportional increase in surface molecules. The MFI values were standardised to enable comparison of MFIs of T cells from different donors.

Figure 12 shows that on CD4 T cells, downregulation of CD3 was stronger the further these T cells were differentiated from T_N to T_{EMRA} cells. The amount of surface CD3 was significantly lower on CD4 T_{CM} , T_{EM} and T_{EMRA} cells compared to T_N cells ($p < 0.001$ for all). Downregulation of CD4 was observed on CD4 T_{EMRA} cells ($p < 0.001$) only, it was not found on T_{CM} and T_{EM} cells compared to CD4 T_N cells (Figure 12). There were no differences between the three age groups in terms of CD3 or CD4 downregulation (data not shown).

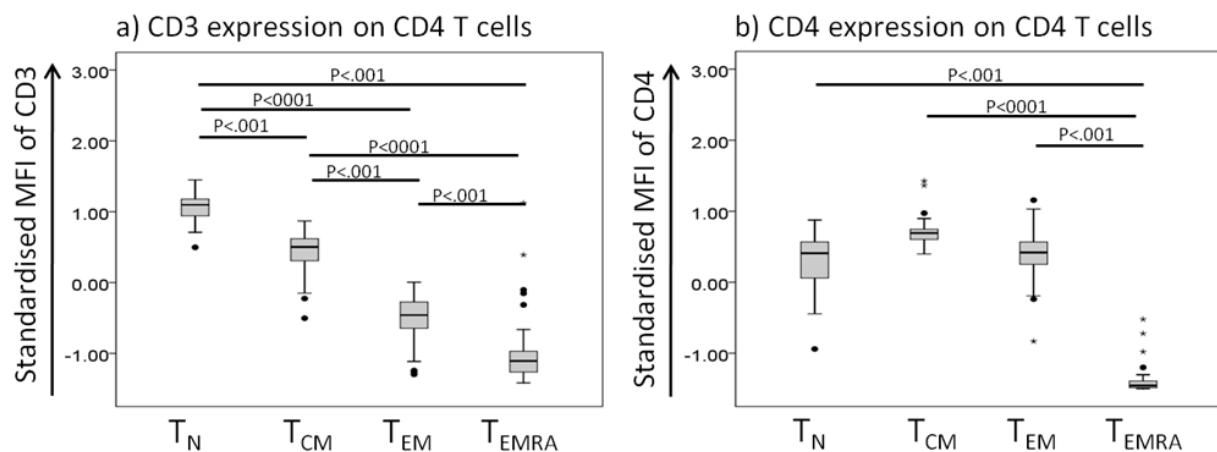


Figure 12: **CD3 and CD4 expression on CD4 T cells with advancing differentiation.** Box plots show standardised MFIs for a) CD3 expression and b) CD4 expression on CD4 T_N , T_{CM} , T_{EM} and T_{EMRA} cells (n=50).

Figure 13 shows the downregulation of CD3 and CD8 on the surface of differentiated CD8 T cells. In a similar manner as described for CD4 T cells, CD3 was decreased on CD8 T_{CM} , T_{EM} and T_{EMRA} cells ($p < 0.001$ for all) as compared to CD8 T_N cells. Surface CD8 was downregulated on CD8 T_{CM} cells as well as T_{EM} and T_{EMRA} cells ($p < 0.001$ for all), as compared to CD8 T_N cells. There were no differences between the three age-groups (data not shown).

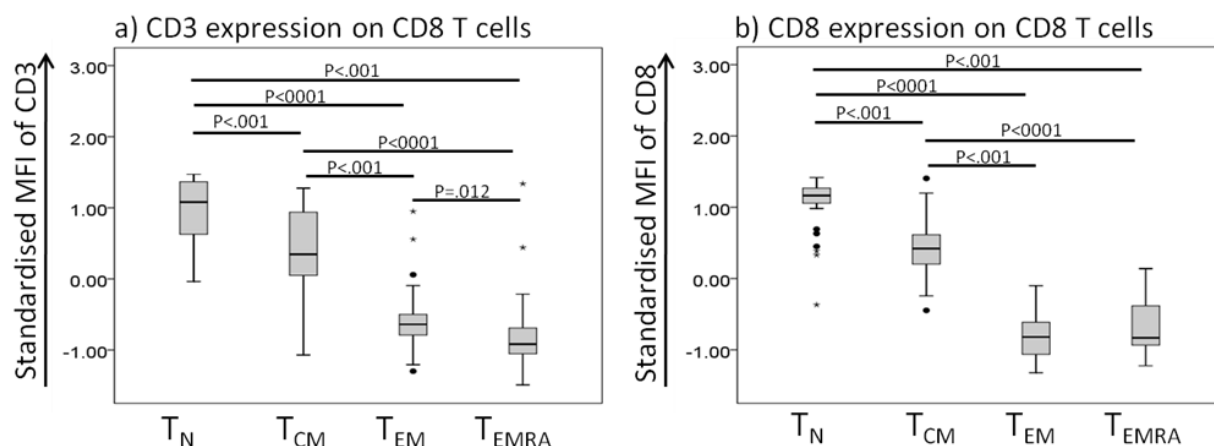


Figure 13: **CD3 and CD8 expression on CD8 T cells with advancing differentiation.** Box plots show standardised MFIs for a) CD3 expression and b) CD8 expression on CD8 T_N, T_{CM}, T_{EM} and T_{EMRA} cells (n=50).

To summarise, these results show that the memory T cell subset distribution changed towards a more differentiated phenotype with advanced age. These changes were more pronounced in the CD8 T cell subset than the CD4 T cell subset which correlates with previous reports [94, 114, 116, 117]. The surface expression of CD3, CD4 and CD8 was decreased on differentiated T cells compared to T_N cells. The downregulation of CD3, CD4 and CD8 was greater the more differentiated the T cells were.

6.3.2 Influence of CMV infection on memory T cell subsets

In order to characterise the differences in the memory T cell subsets induced by CMV infection, the expression of CD27 and CD45RA was analysed on PBMC from CMV-seropositive (n=24) and CMV-seronegative (n=25) individuals by flow cytometry. The CMV-serostatus for one donor couldn't be determined, this donor was therefore excluded from CMV-related analyses.

CMV infection appeared to alter the entire T cell population of infected individuals compared to uninfected donors (Figure 14, Table 10). On average, CMV-seropositive people had, in proportion, significantly more CD4 T_{EM} and T_{EMRA} cells than CMV-negative people (Figure 14, p<0.001 for both). The same was observed for absolute numbers of CD4 T_{EM} and T_{EMRA} cells (supplemental Figure 57, p<0.001 for both).

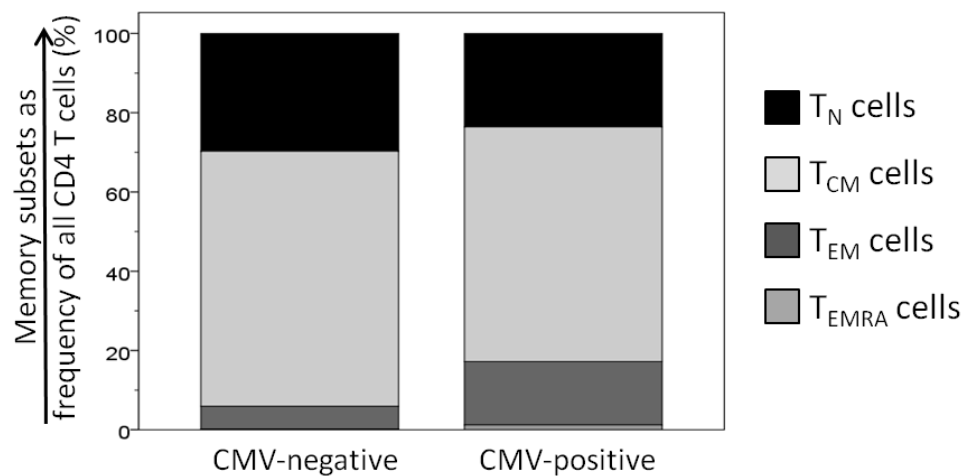


Figure 14: **Distribution of the four memory subsets in all CD4 T cells.** Stacked bars display mean frequencies of the four memory CD4 T cell subsets for CMV-negative (n=25) and CMV-positive donors (n=24).

	CMV-negative	CMV-positive	p
N	25	24	
% CD4 T _N cells median (range)	24 (5-55)	22 (2-57)	ns
% CD4 T _{CM} cells median (range)	62 (41-84)	57 (27-82)	ns
% CD4 T _{EM} cells median (range)	6 (2-15)	11 (4-40)	<0.001
% CD4 T _{EMRA} cells median (range)	0.1 (0-3)	0.5 (0-7)	<0.001
CD4 T _N cell count (10 ³ /ml blood) median (range)	147 (27-453)	215 (22-548)	ns
CD4 T _{CM} cell count (10 ³ /ml blood) median (range)	435 (160-812)	519 (136-1329)	ns
CD4 T _{EM} cell count (10 ³ /ml blood) median (range)	40 (14-169)	115 (34-394)	<0.001
CD4 T _{EMRA} cell count (10 ³ /ml blood) median (range)	1 (0-16)	5 (1-95)	<0.001

Table 10: Memory CD4 T cell subsets in CMV-seronegative and CMV-seropositive donors

CMV-positive and CMV-negative donors were also analysed separately for changes of their memory T cell subset distribution with age. CMV-seronegative people showed a trend towards enhanced frequencies of CD4 T_{CM} cells with advancing age, while frequencies of CD4 T_{EM} cells remained constant. This was in contrast to CMV-seropositive individuals who showed constant frequencies of CD4 T_{CM} cells and a trend towards increasing frequencies of CD4 T_{EM} cells with age (Figure 15). In both infected and uninfected people, the frequencies of T_N cells tended to diminish with age while the frequencies of CD4 T_{EMRA} cells remained constant.

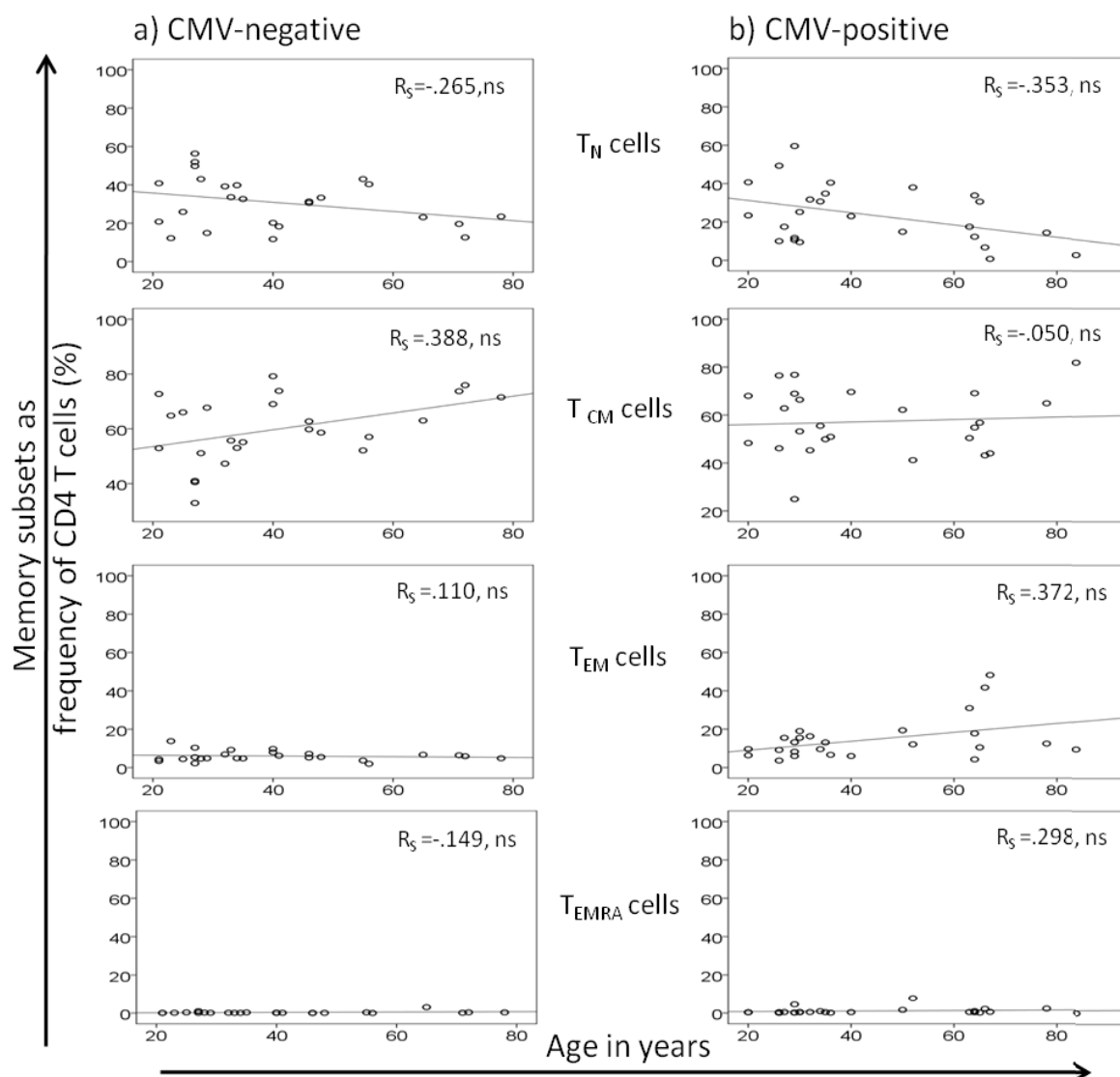


Figure 15: **Correlation of memory CD4 T cell subsets with age and CMV status.** Charts represent the distribution of all CD4 T cells in T_N , T_{CM} , T_{EM} and T_{EMRA} cells plotted against age in a) CMV-seronegative donors (n=25) and b) CMV-seropositive donors (n=24).

Analysis of the CD8 T cell subset demonstrated that CMV-infected donors had proportionally significantly less CD8 T_N cells and more CD8 T_{EM} cells than CMV-negative donors (Figure 16, Table 11, $p=0.014$ and $p=0.022$ respectively). It was noted that there was a trend for CMV-positive people to have more CD8 T_{EMRA} cells than CMV-negative people but this was not statistically significant. In absolute terms, CMV-seropositive donors had significantly more CD8 T_{EM} and T_{EMRA} cells than CMV-seronegative donors (supplemental Figure 57, $p=0.027$ and $p=0.006$, respectively).

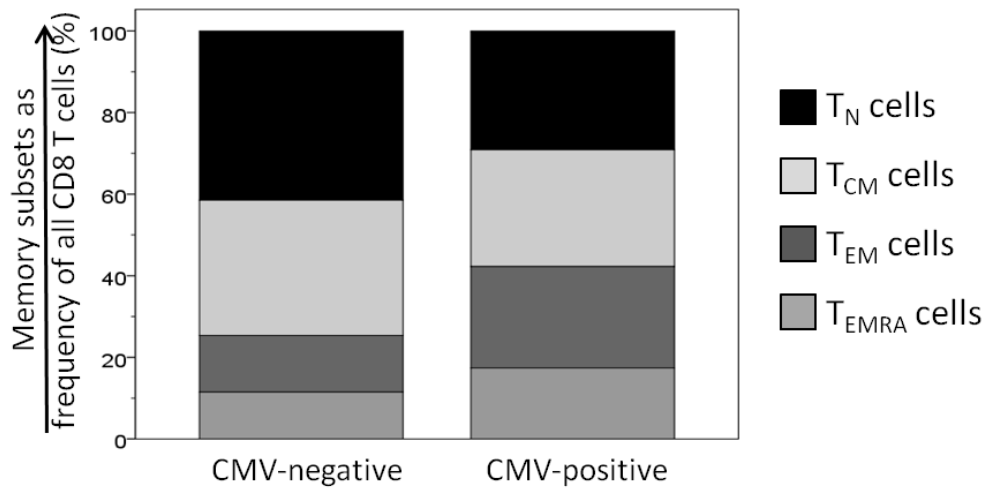


Figure 16: **Distribution of the four memory subsets in all CD8 T cells.** Stacked bars display mean frequencies of the four memory CD8 T cell subsets for CMV-negative (n=25) and CMV-positive donors (n=24).

	CMV-negative	CMV-positive	p
N	25	24	
% CD8 T _N cells median (range)	40 (9-65)	30 (1-65)	0.014
% CD8 T _{CM} cells median (range)	33 (15-51)	25 (2-64)	ns
% CD8 T _{EM} cells median (range)	13 (4-31)	19 (4-75)	0.022
% CD8 T _{EMRA} cells median (range)	6 (1-37)	10 (1-55)	ns
CD8 T _N cell count (10 ³ /ml blood) median (range)	176 (32-445)	164 (5-395)	ns
CD8 T _{CM} cell count (10 ³ /ml blood) median (range)	146 (46-541)	155 (9-599)	ns
CD8 T _{EM} cell count (10 ³ /ml blood) median (range)	47 (14-255)	109 (15-658)	0.027
CD8 T _{EMRA} cell count (10 ³ /ml blood) median (range)	30 (7-210)	68 (17-729)	0.006

Table 11: CD8 memory T cell subsets in CMV-seronegative and CMV-seropositive donors

Both infected and uninfected donors showed significant decreasing frequencies of CD8 T_N cells ($R_s = -0.721$, $p < 0.001$ and $R_s = -0.528$, $p = 0.002$ respectively) and significant increasing frequencies of CD8 T_{EMRA} cells with age ($R_s = 0.515$, $p = 0.008$ and $R_s = -0.431$, $p = 0.032$ respectively). The frequency of CD8 T_{EM} cells was significantly increased ($R_s = 0.484$, $p = 0.014$) in CMV-infected donors with advanced age while the frequency of CD8 T_{CM} cells was decreased (Figure 17). In uninfected people, the frequency of CD8 T_{CM} and T_{EM} cells increased significantly ($R_s = 0.405$, $p = 0.044$ and $R_s = 0.468$, $p = 0.018$ respectively).

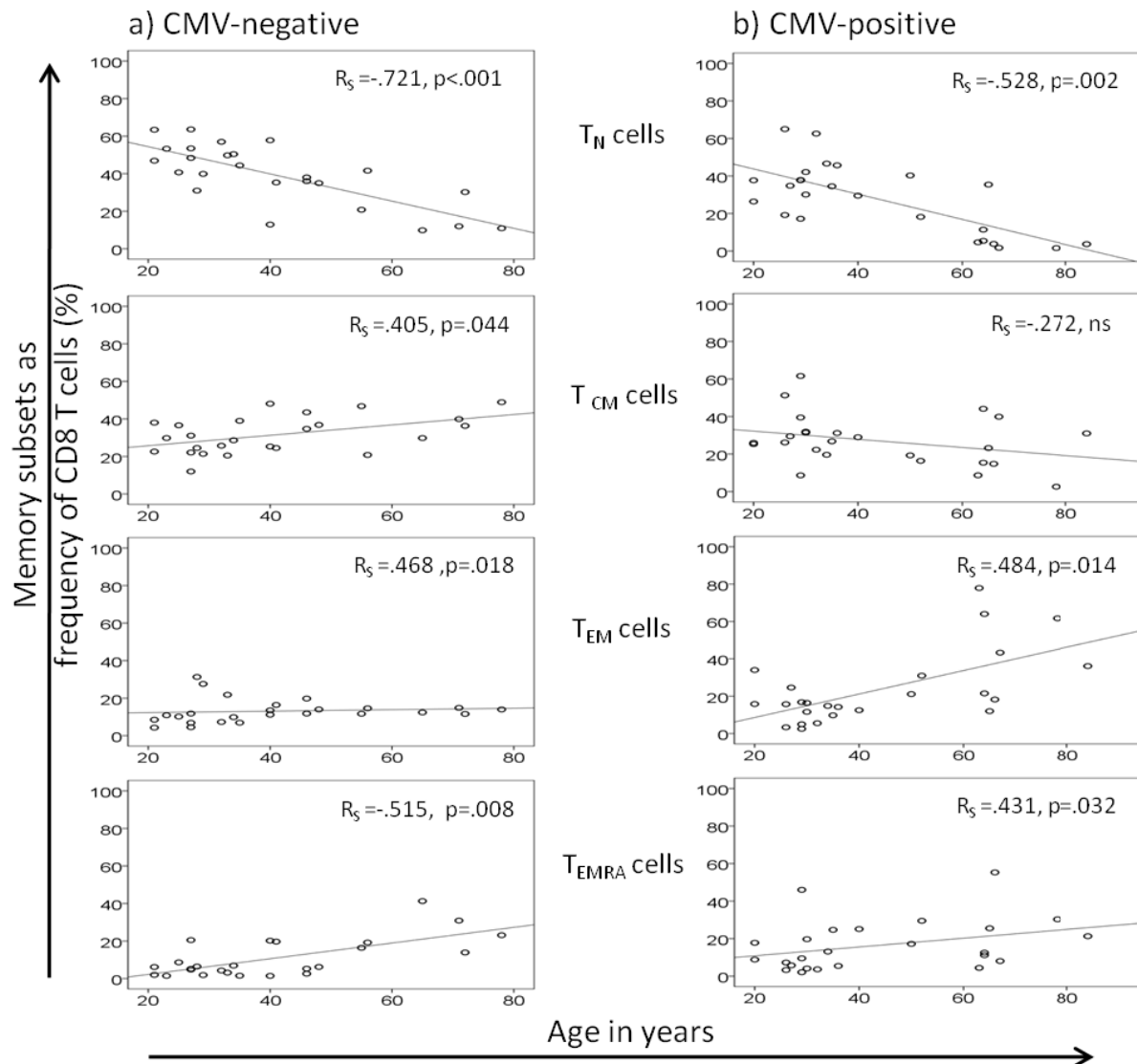


Figure 17: **Correlation of memory CD8 T cell subsets with age and CMV status.** Charts represent the distribution of all CD8 T cells in T_N , T_{CM} , T_{EM} and T_{EMRA} cells plotted against age in a) CMV-seronegative donors ($n=25$) and b) CMV-seropositive donors ($n=24$).

To summarise, CMV-infected donors in this study had more CD4 and CD8 T_{EM} and T_{EMRA} cells and less CD8 T_N cells, resulting in a more differentiated phenotype overall than in CMV-seronegative donors. CMV-seropositive donors showed increasing numbers of T_{EM} cells with advancing age while T_{CM} cells increased in CMV-seronegative donors with age. In general, CMV-seropositive donors had a more differentiated T cell distribution than CMV-seronegative donors and this became more pronounced with age.

6.4 CMV-specific T cell response

6.4.1 Age and the CMV-specific T cell response

To analyse the phenotype and functionality of CMV-specific T cells, multicolour flow cytometric experiments were performed. Several functional read-outs were combined to increase both sensitivity of detection and resolution of functional profiles: CD40L, IFN γ , IL2, and TNF. PBMC were stimulated with CMV-spanning peptide pools (IE1 or pp65) and T cells that produced one or more of the activation markers after background subtraction were considered responding T cells. These responding T cells are termed pp65- or IE1-specific T cells.

All CMV-seropositive donors, but none of the CMV-seronegative donors had pp65 and/or IE1-specific T cell responses above background levels. CMV-seropositive donors had relatively and absolutely more unstimulated CD8 T cells producing TNF than CMV-seronegative donors ($p=0.05$ and $p=0.02$ respectively, data not shown).

The correlation of frequencies and absolute numbers of pp65 and IE1-specific T cells per ml blood in CMV-positive donors is shown in Figure 18. The absolute numbers of pp65-specific CD4 T cells per ml blood and the frequency of pp65-specific CD4 T cells as percentage of all CD4 T cells were very closely correlated (Figure 18, $R_s = 0.952$, $p < 0.001$). A similar correlation was observed for absolute numbers and frequencies of both pp65 and IE1-specific CD8 T cells (Figure 18, $R_s = 0.959$, $p < 0.001$ and $R_s = 0.961$, $p < 0.001$ respectively).

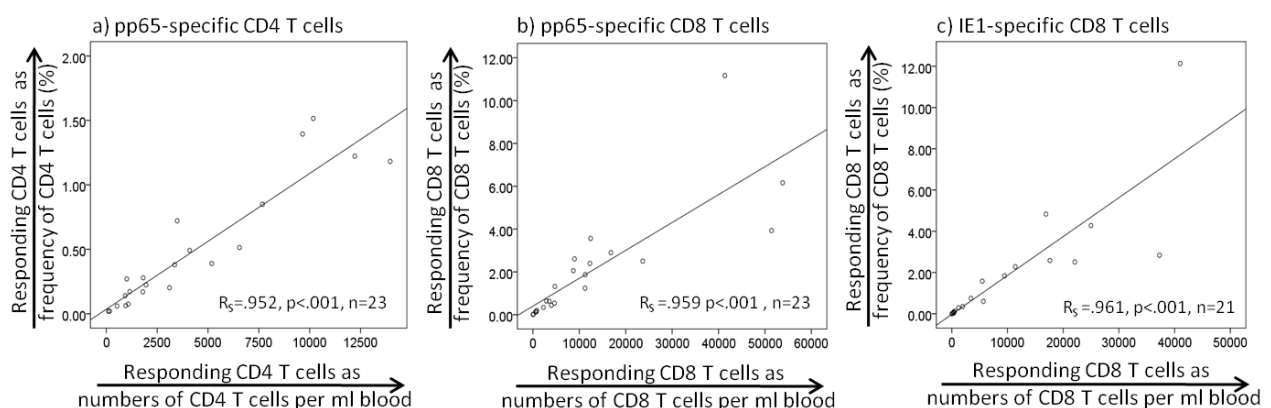


Figure 18: Absolute numbers of cells per ml blood and the frequency of reacting cells after stimulation with either CMV peptide pools are correlated. Frequencies and absolute numbers per ml blood of a) pp65-specific CD4 T cells, b) pp65-specific CD8 T cells and c) IE1-specific CD8 T cells for all donors are represented by dots.

The number of pp65-specific T cells as a percentage of CD4 T cells ranged from 0.02-1.52% (n=23, median 0.28%) and in absolute terms the number of activated CD4 T cells was 103-13956 cells per ml blood (n=23, median=2530 cells per ml blood, Table 14). The respective numbers of IE1-specific CD4 T cells were 0.01-0.4% of all CD4 T cells (n=22, median 0.06%) and 53-4616 cells per ml blood (n=21, median 460 cells per ml blood).

Both the frequencies and absolute numbers of CD4 T cells responding to stimulation with pp65 increased with age and this correlated with previous studies (Figure 19 and supplemental Figure 58)[115, 118, 119]. The absolute numbers, but not the frequencies, of pp65-specific CD4 T cells per ml blood correlated significantly with age ($R_s=0.512$, $p=0.015$). Nevertheless, comparison of the three age groups revealed significant differences between young and old people in terms of the frequency and absolute number of CD4 T cells responding to stimulation with pp65 (Figure 20 and supplemental Figure 59, $p=0.026$ and $p=0.012$, respectively).

There were no significant correlations between IE1-specific CD4 T cell numbers or frequencies and the age of the donor (Figure 19, Figure 20, supplemental Figure 58 and Figure 59). Since the IE1-specific CD4 T cell response was very small in most donors it was excluded from further analysis.

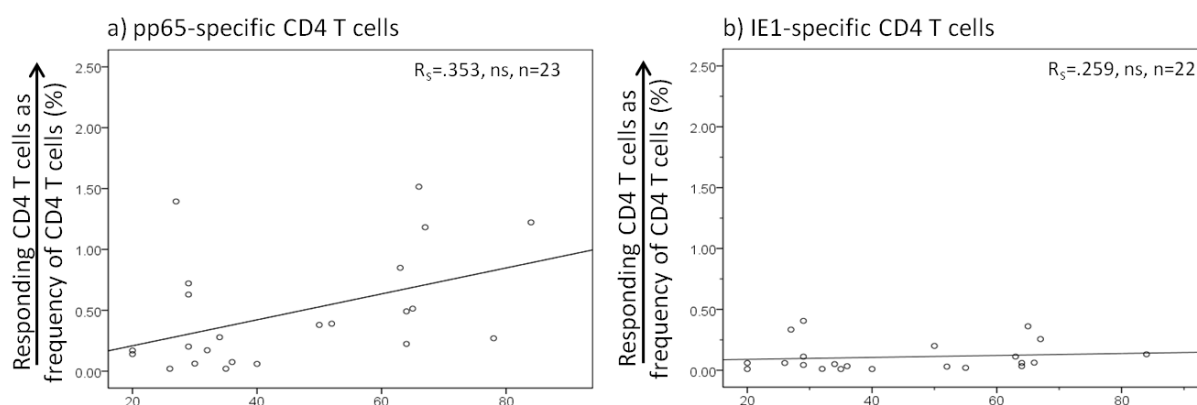


Figure 19: **Frequencies of CMV-specific CD4 T cells correlated with age.** Scatter plots show the distribution of frequencies of a) pp65-specific CD4 T cells and b) IE1-specific CD4 T cells and age of the donors.

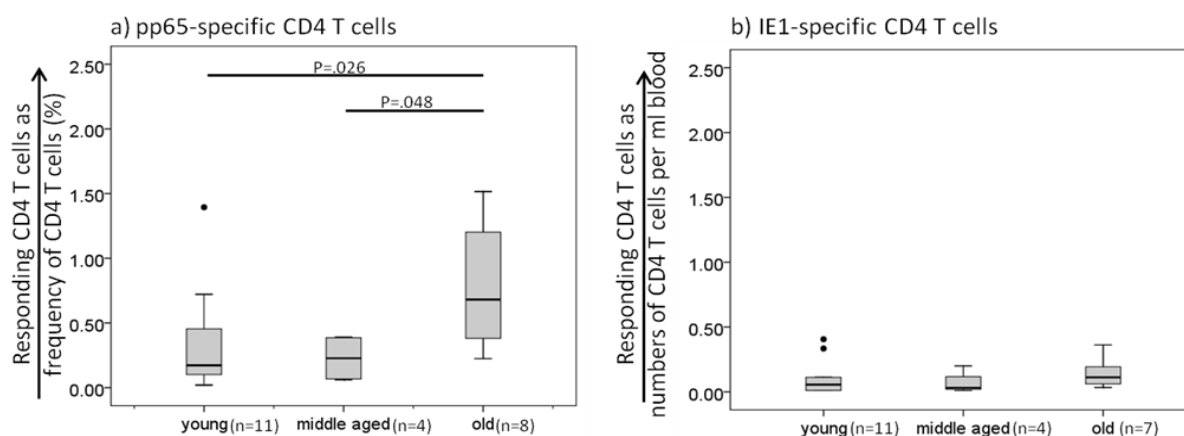


Figure 20: **Frequencies of pp65 and IE1-specific CD4 T cells in the three age groups.** Box plots represent frequencies of a) pp65-specific CD4 T cells and b) IE1-specific CD4 T cells in young, middle-aged and old donors.

Since the entire memory T cell subset distribution changed with age, the memory subset distribution of CMV-specific T cells were analysed to determine if they also change with age. The results showed that the distribution of memory T cell subsets of pp65-specific CD4 T cells differed in the three age groups. In terms of percentage of activated CD4 T cells, old donors had significantly less CD4 T_{CM} cells and more CD4 T_{EM} cells than young donors (Figure 21, $p=0.027$ and $p=0.043$, respectively). In absolute counts this difference was significant for CD4 T_{EM} cells ($p=0.006$), but not for CD4 T_{CM} cells (supplemental Figure 60).

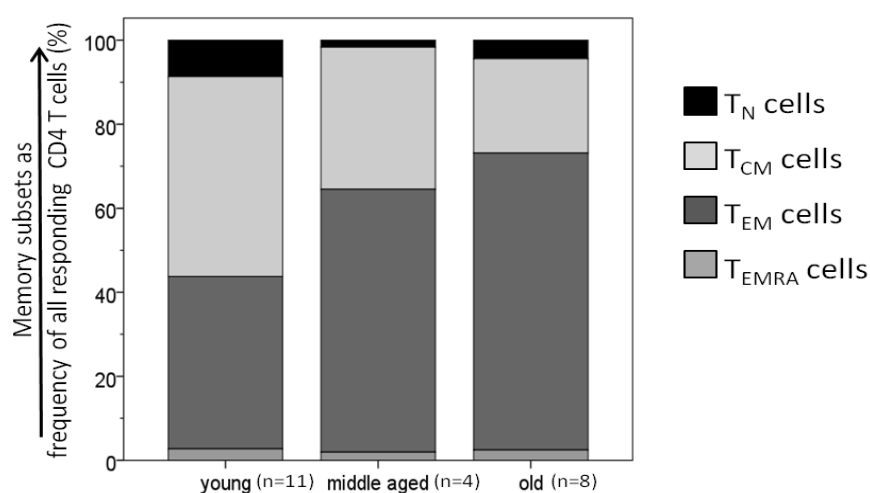


Figure 21: **Distribution of the four memory subsets in pp65-specific CD4 T cells in the three age groups.** Stacked bars display mean frequencies of the four memory CD4 T cell subsets for young, middle-aged and old donors.

The results also indicated that the composition of the memory T cell subsets of responding T cells in CMV-positive donors correlated with age and with the response size following stimulation with pp65-spanning peptide pools (Figure 22). The frequencies of pp65-specific CD4 T_N ($R_S = -0.475$, $p=0.025$) and T_{CM} cells ($R_S = -0.491$, $p=0.020$) were significantly diminished while the frequencies of pp65-specific CD4 T_{EM} cells ($R_S = 0.525$, $p=0.012$) expanded with increasing response size to pp65 stimulation. The frequencies of pp65-specific CD4 T_{EMRA} cells were not significantly correlated with the pp65-specific response size.

In addition, similar patterns were seen when the entire memory CD4 T cell distribution was correlated with the pp65-specific T cell response size. The frequencies of all CD4 T_N cells (Figure 22, $R_S = -0.497$, $p=0.016$) were significantly diminished with increasing pp65-specific T cell response size while the frequencies of all CD4 T_{EM} cells were expanded ($R_S = 0.446$, $p=0.033$). The frequencies of all CD4 T_{CM} and T_{EMRA} cells were not significantly correlated with the pp65-specific response size. The degree of advanced T-cell memory differentiation within the pp65-specific response, but also in general, therefore, seems to depend on the size of the CMV-specific T cell response with small responses having little effect but large responses a big effect.

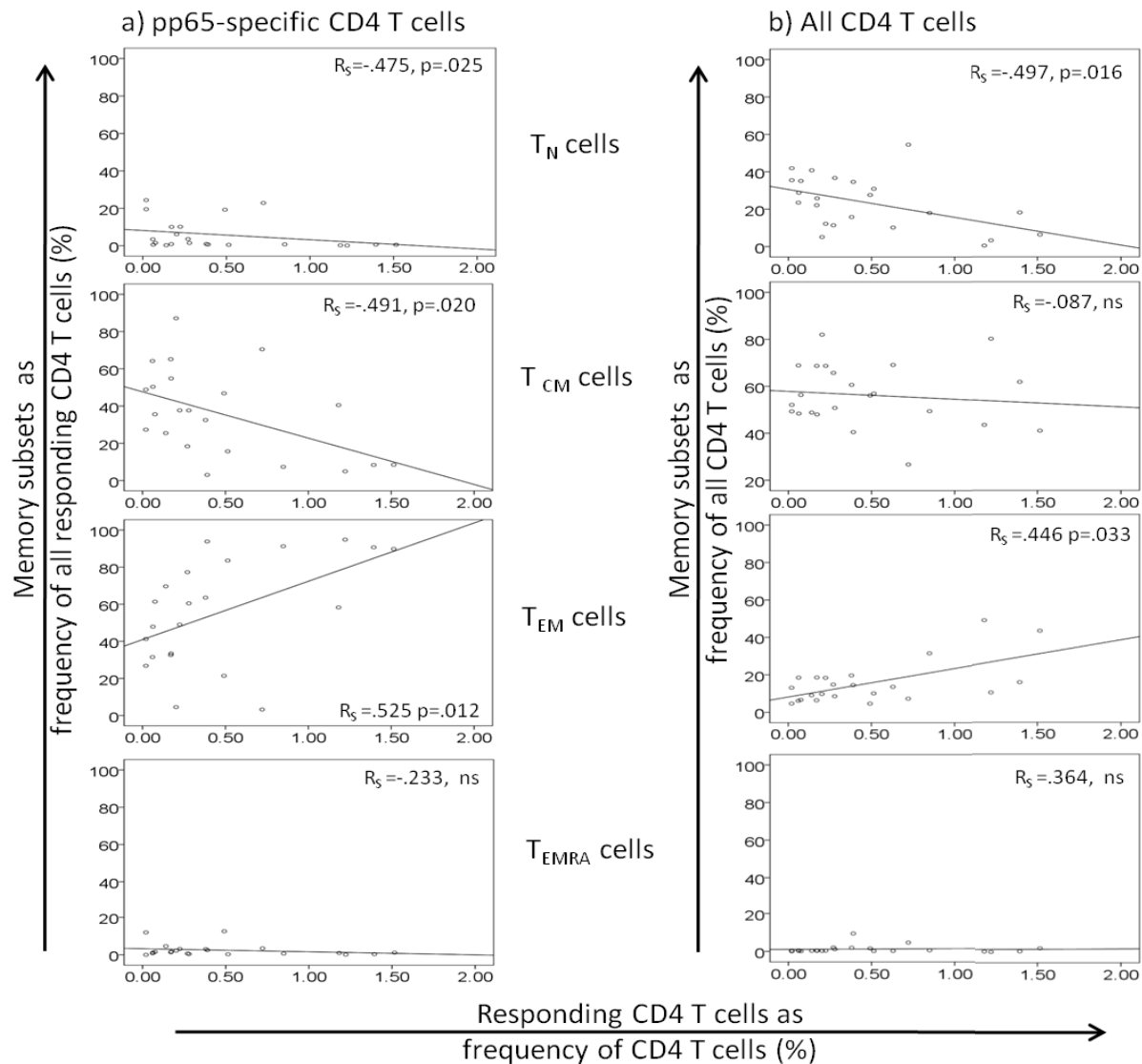


Figure 22: **Correlation of response size and memory subsets of pp65-specific and all CD4 T cells.** Scatter plots represent the distribution of a) pp65-specific CD4 T cells and b) all CD4 T cells in T_N , T_{CM} , T_{EM} and T_{EMRA} cells correlated to the response size to pp65 stimulation (n=23).

Equivalent analyses were carried out for CD8 T cells. The frequencies of pp65-specific CD8 T cells ranged from 0.01-11.17% (n=24, median=1.28%) of all CD8 T cells and 39-53800 cells per ml blood (n=23, median=6729 cells per ml blood, supplemental Table 14). This is in accordance with previous studies which showed that the CD8 T cell response after viral infection is, in most individuals, larger than the CD4 T cell response (rev. in [120]). The frequencies of responding CD8 T cells after IE1 stimulation ranged from 0.0002-12.13% (n=21, median=0.75%) of all CD8 T cells and 149-40996 cells per ml blood (n=20, median=5471 cells per ml blood).

Frequencies and absolute numbers of CD8 T cells that responded to stimulation with pp65 increased with age (Figure 23 and supplemental Figure 61). The frequencies of pp65-specific CD8 T cells increased significantly with age ($R_s=0.427$, $p=0.037$) whereas the increase in absolute numbers of pp65-specific CD8 T cells was not statistically significant. Nevertheless, comparison of the three age groups revealed significant differences between the frequencies of responding CD8 T cells in young and old people and between absolute numbers in middle-aged and old people (Figure 24 and supplemental Figure 62, $p=0.016$ and $p=0.048$ respectively). Frequencies and absolute numbers of IE1-specific CD8 T cells were slightly higher in middle-aged and old people but differences did not reach statistical significance (Figure 23, Figure 24, supplemental Figure 61 and supplemental Figure 62).

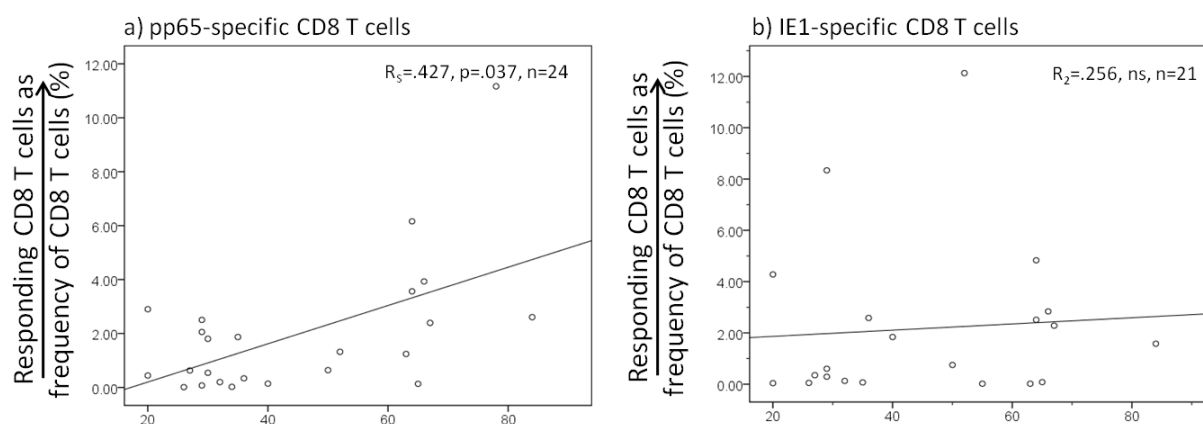


Figure 23: **Frequencies of pp65 and IE1-specific CD8 T cells correlated with age.** Scatter plots show the distribution of frequencies of a) pp65-specific CD8 T cells and d) IE1-specific CD8 T cells and age of the donors.

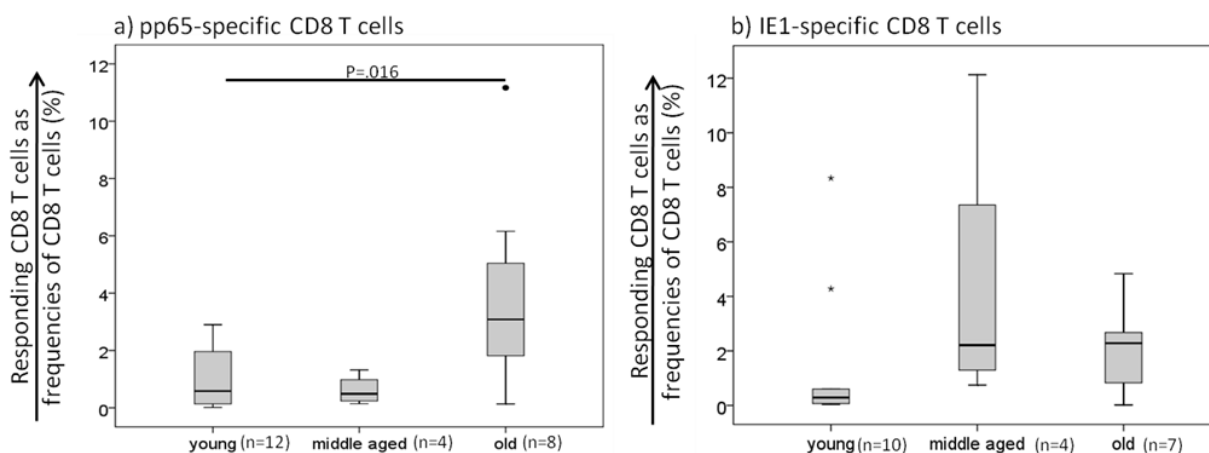


Figure 24: **Frequencies of pp65 and IE1-specific CD8 T cells in the three age groups.** Box plots represent frequencies of a) pp65-specific CD8 T cells and b) IE1-specific CD8 T cells in young, middle-aged and old people.

The memory T cell subsets of CMV-specific CD8 T cells differed in the three age groups. Old donors had in proportion significantly less pp65-specific CD8 T_{CM} cells than young donors (Figure 25, $p=0.004$). The differences in frequencies of pp65-specific CD8 T_N, T_{EM} and T_{EMRA} cells were not significant. In contrast, old donors had a significantly more absolute numbers of pp65-specific CD8 T_{EM} and T_{EMRA} cells than young (supplemental Figure 60, $p=0.006$ and $p=0.012$ respectively) or middle-aged donors ($p=0.028$ and $p=0.048$ respectively).

Middle-aged and old donors had, in proportion significantly, less IE1-specific CD8 T_{CM} cells ($p=0.003$ and 0.042 respectively) and significantly more IE1-specific CD8 T_{EM} cells than young donors (Figure 25, $p=0.05$ and $p=0.042$ respectively). Middle-aged donors also had more absolute numbers of T_{EM} cells than young donors (supplemental Figure 60, $p=0.016$). The differences in T_N and T_{EMRA} IE1-specific CD8 T cells were not significant.

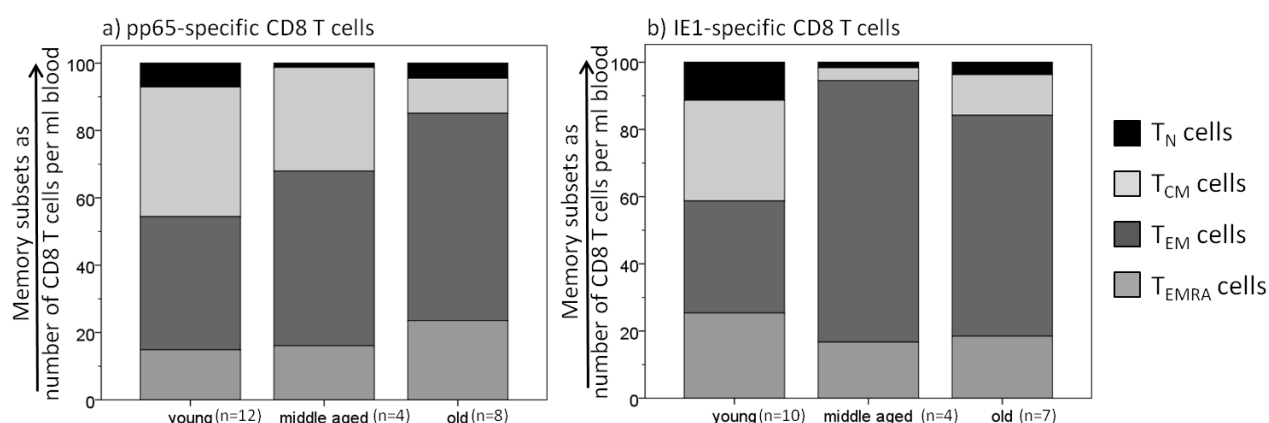


Figure 25: **Distribution of the four memory subsets in CMV-specific CD8 T cells in the three age groups.** Stacked bars display mean frequencies of the four memory T cell subsets for a) pp65-specific CD8 T cells and b) IE1-specific CD8 T cells in young, middle-aged and old people.

The memory T cell subset composition of responding T cells in CMV-positive donors was not only correlated with the age, but also with the response size to stimulation with pp65-spanning peptide pools (Figure 26). The frequencies of pp65-specific CD8 T_N ($R_S = -0.669$, $p=0.001$) and T_{CM} cells ($R_S = -0.523$, $p=0.012$) were significantly diminished with increasing response size to pp65 stimulation. Frequencies of pp65-specific CD8 T_{EM} and T_{EMRA} cells increased with increasing response size to pp65 stimulation but this was not statistically significant.

Similar tendencies were seen when the entire CD8 T cell population was correlated with the CMV-specific T cell response size. The frequencies of all CD8 T_N cells ($R_S = -0.787$, $p<0.001$)

were significantly diminished while the frequencies of all CD8 T_{EM} ($R_S = 0.538$, $p=0.007$) and T_{EMRA} cells ($R_S = 0.429$, $p=0.037$) expanded with increasing response size to pp65 stimulation. The frequencies of all CD8 T_{CM} cells decreased with the pp65-specific response size but this was not significant. The frequencies of IE1-specific CD8 T_N cells was significantly diminished and frequencies of IE1-specific CD8 T_{EM} cells increased with increasing response size to IE1 stimulation (Figure 27, $R_S = -0.642$, $p=0.003$ and $R_S = -0.470$, $p=0.042$ respectively). Unlike the size of the pp65 specific CD8 T cell response, the size of the IE-1 specific CD8 T cell response did not affect the memory subset distribution within the entire CD8 T cell compartment. In brief, the bigger the CD8 T cell response to pp65 the bigger was the proportion of T cells with an advanced memory phenotype in the entire (including non-CMV-specific) T cell compartment.

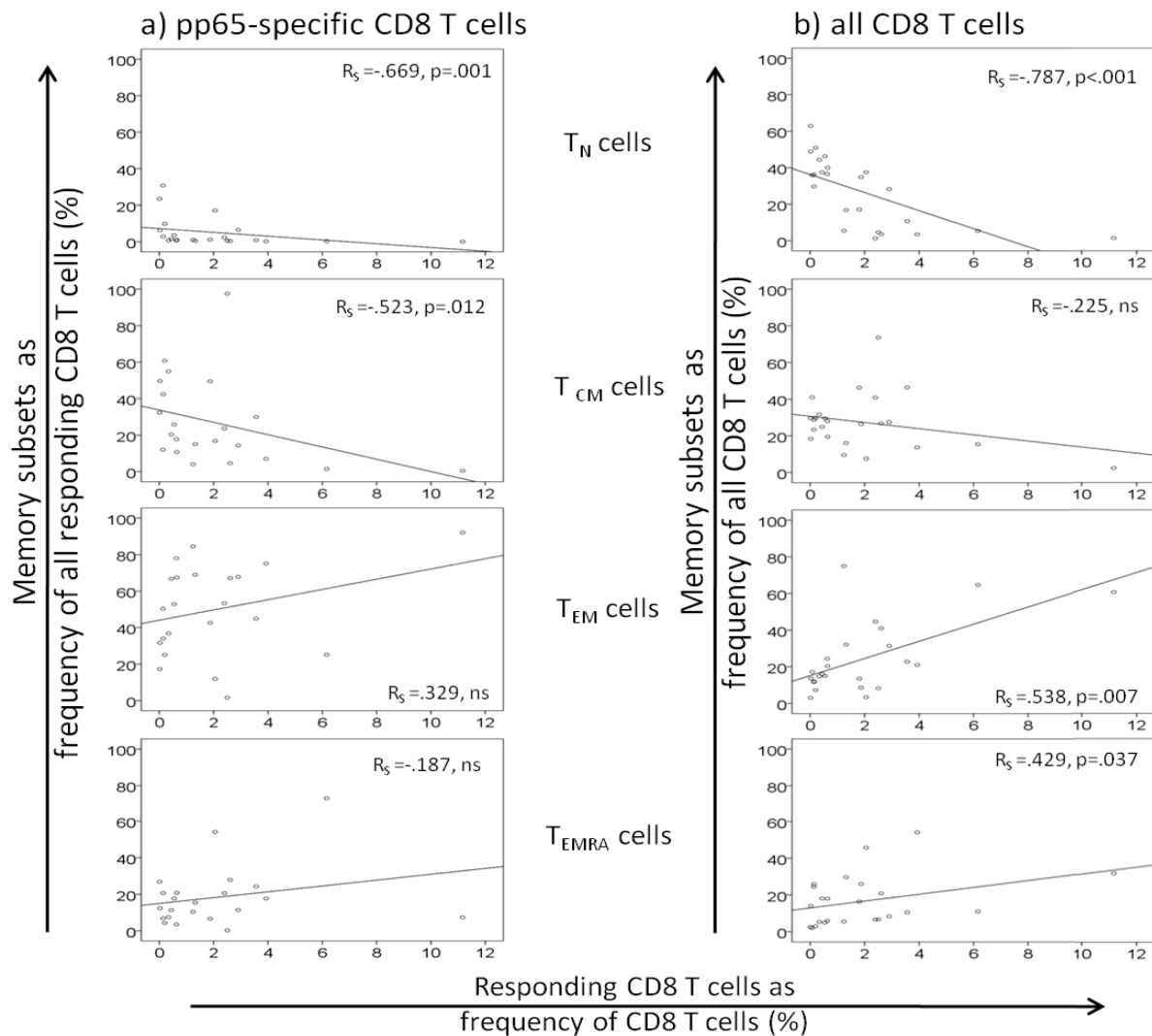


Figure 26: **Correlation of pp65-specific response size and memory subsets of pp65-specific and all CD8 T cells.** Scatter plots represent the distribution of responding a) pp65-specific CD8 T cells and b) all CD8 T cells in T_N , T_{CM} , T_{EM} and T_{EMRA} cells correlated with the pp65-specific T cell response size ($n=24$).

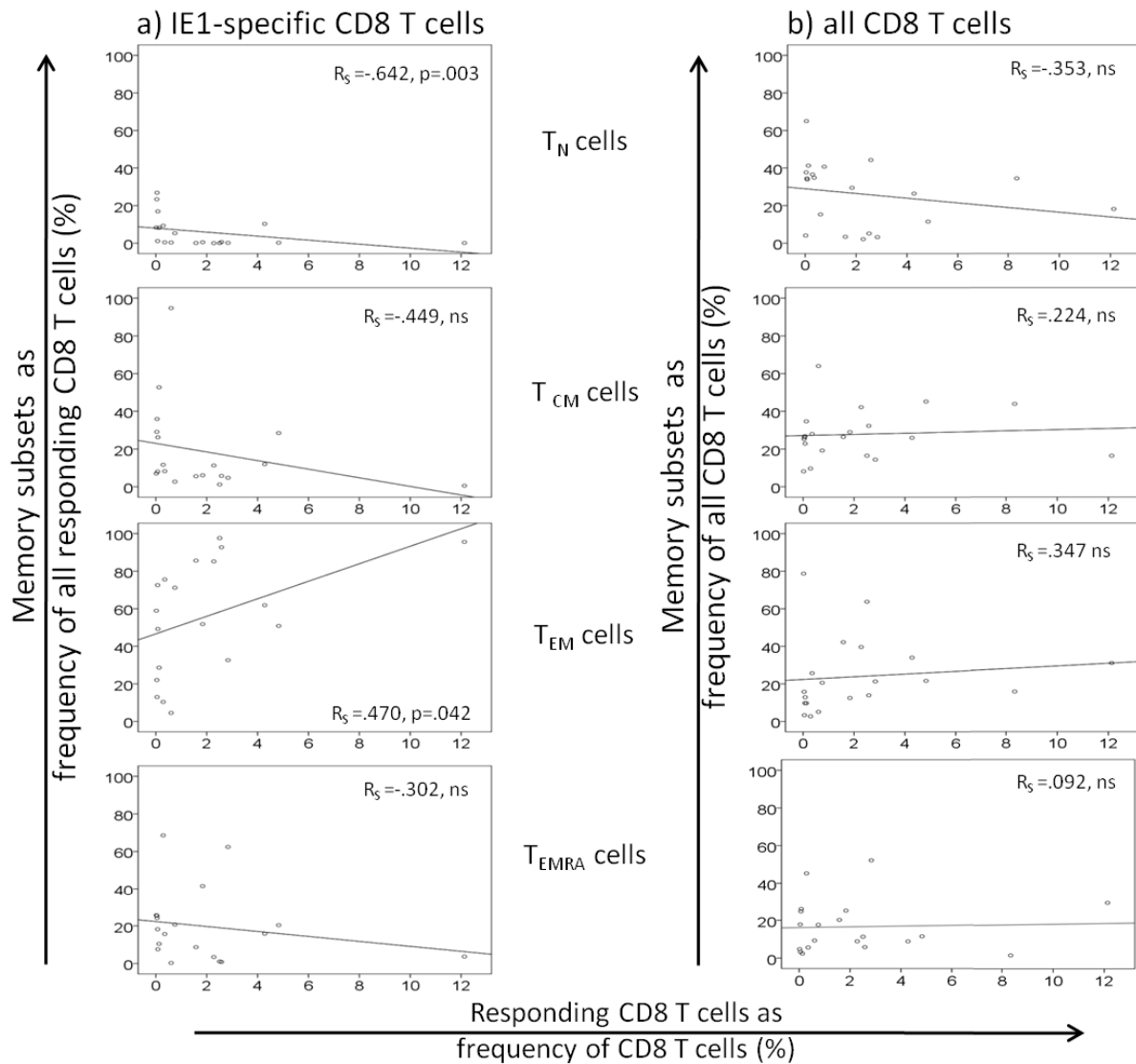


Figure 27: **Correlation of IE1-specific response size and memory subsets of IE1-specific and all CD8 T cells.** Scatter plots represent the distribution of responding a) IE1-specific CD8 T cells and b) all CD8 T cells in T_N , T_{CM} , T_{EM} and T_{EMRA} cells correlated to the IE-specific T cell response size (n=20).

In concordance with Gamadia *et al.*(2004), this study illustrated that the frequencies of pp65-specific CD8 T cells were linearly correlated with the frequencies of CD27⁺ CD8 T cells (Figure 28, $R_s = 0.540$, $p = 0.009$) [121]. The same correlation was observed between pp65-specific CD4 T cells and CD27⁺ CD4 T cells ($R_s = 0.5620$, $p = 0.002$). No significant correlation was seen between the frequencies of IE1-specific CD8 T cells and the frequencies of CD27⁺ CD8 T cells.

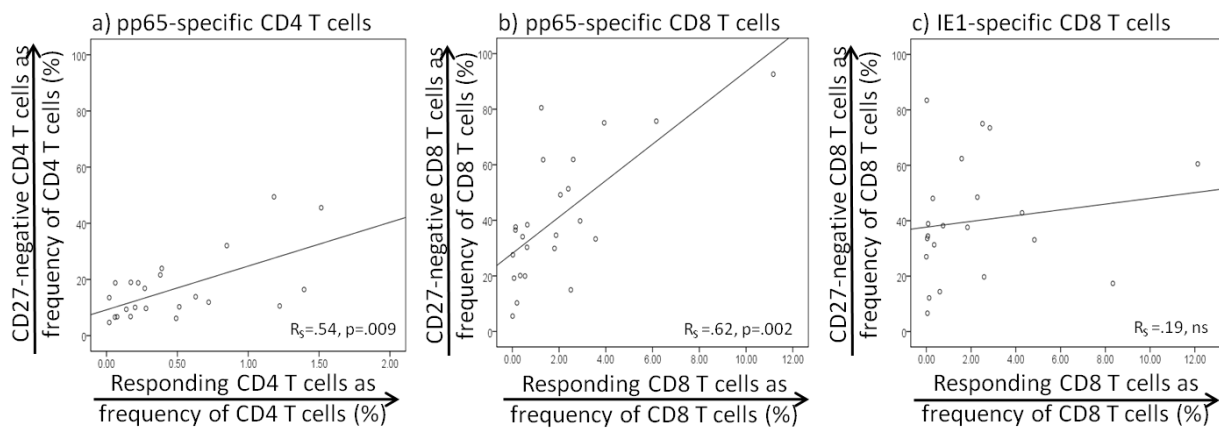


Figure 28: **Correlation of response size and CD27⁻ T cells.** Data are presented as dots correlating the frequencies of a) pp65-specific CD27⁻ CD4 T cells (n=23), b) pp65-specific CD27⁻ CD8 T cells (n=25) and c) IE1-specific CD27⁻ CD8 T cells (n=20) to frequencies of responding T cells.

The proteins pp65 and IE1 are selective and dominant CMV antigens. The changes of the T cell response as a result of ageing, measured with these antigens may not be representative of other CMV antigens or antigens derived from other sources. It was therefore of interest to use PPD as recall antigen to stimulate T cells.

The number of PPD-specific CD4 T cells ranged from 0.03-0.72% (n=24, median=0.17%) of all CD4 T cells and 257-5655 cells per ml blood (n=24, median=1390 cells per ml blood). The frequencies of PPD-specific CD8 T cells ranged from 0-0.18% (n=24, median=0.02%) of all CD8 T cells and 11-1578 cells per ml blood (n=24, median=137 cells per ml blood, supplemental Table 14). Since the PPD-specific CD8 T cell response was very small in most donors, it was excluded from further analysis. There were no significant differences between the three age groups regarding the frequency, absolute numbers or memory subsets of activated PPD-specific CD4 T cells (Figure 29 and supplemental Figure 63).

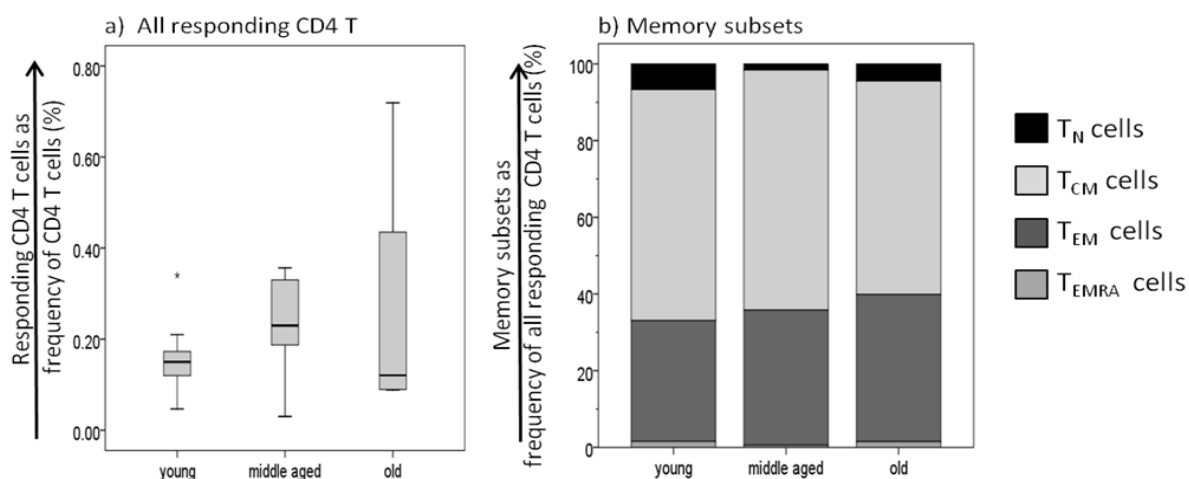


Figure 29: **Frequencies and memory subsets of responding CD4 T cells in three age groups after PPD stimulation.** a) Box plots represent the frequencies of responding CD4 T cells and b) stacked bars display frequencies of the four memory subsets in responding CD4 T cells after stimulation with PPD in young (n=11), middle-aged (n=9) and old (n=4) donors.

In addition, a polyclonal stimulus, OKT3, was used to investigate T cells of different antigen specificities. OKT3 induces polyclonal T cell stimulation by binding to CD3 on the cell surface [122, 123]. PBMC from all donors responded to stimulation with OKT3 for 16 hours. The number of responding CD4 T cells after OKT3 stimulation ranged from 8.83-62.41% (n=50, median 18.80%) of all CD4 T cells and 19749-410183 cells per ml blood (n=47, median 156686 cells per ml blood). The number of responding CD8 T cells after OKT3 stimulation was smaller than the CD4 T cells and ranged from 1.79-32.8% (n=50, median=8.11%) of all CD8 T cells and 5786-287057 cells per ml blood (n=47, median=45743 cells per ml blood, supplemental Table 14). The frequencies and effector functions of responding CD4 T cells after OKT3 stimulation remained constant throughout the three age groups (Figure 30). The memory subsets in old donors contained proportionally less CD4 T_N and more T_{EMRA} cells than in young donors (p=0.026 and p=0.011 respectively). In absolute terms, the number and memory T cell distribution of CD4 T cells responding to OKT3 stimulation did not differ between the three age groups (supplemental Figure 64).

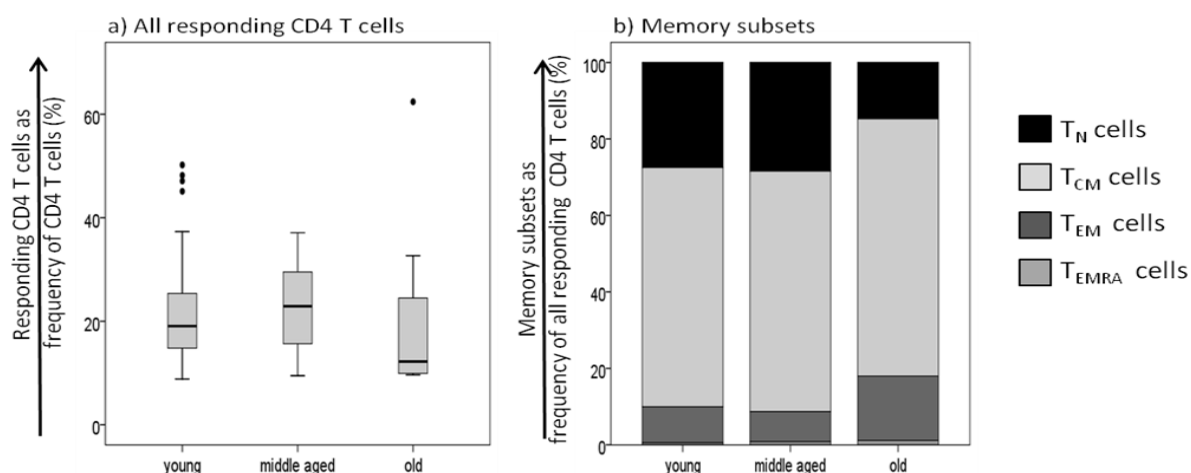


Figure 30: **Frequencies and memory subsets of responding CD4 T cells in three age groups after OKT3 stimulation.** a) Box plots represent the frequencies of responding CD4 T cells and b) stacked bars display frequencies of the four memory subsets in responding CD4 T cells after stimulation with OKT3 in young (n=27), middle-aged (n=11) and old (n=12) donors.

The frequencies of responding CD8 T cells after OKT3 stimulation increased with age (Figure 31). In absolute terms, old people had more CD8 T cells responding to OKT3 stimulation than young people (supplemental Figure 64, $p=0.004$). The memory subset distribution of responding CD8 T cells after OKT3 stimulation was different between young and old donors. Activated CD8 T_N ($p<0.001$) and T_{CM} ($p=0.049$) cell subsets were proportionally smaller, whereas CD8 T_{EMRA} (ns) and T_{EM} ($p=0.038$) cell subsets were bigger in old people compared to young people. In absolute terms, old people had significantly lower numbers of CD8 T_N cells ($p=0.001$) and higher numbers of CD8 T_{EM} and T_{EMRA} cells responding after OKT3 stimulation than young people (supplemental Figure 64, $p=0.002$ and $p=0.001$ respectively).

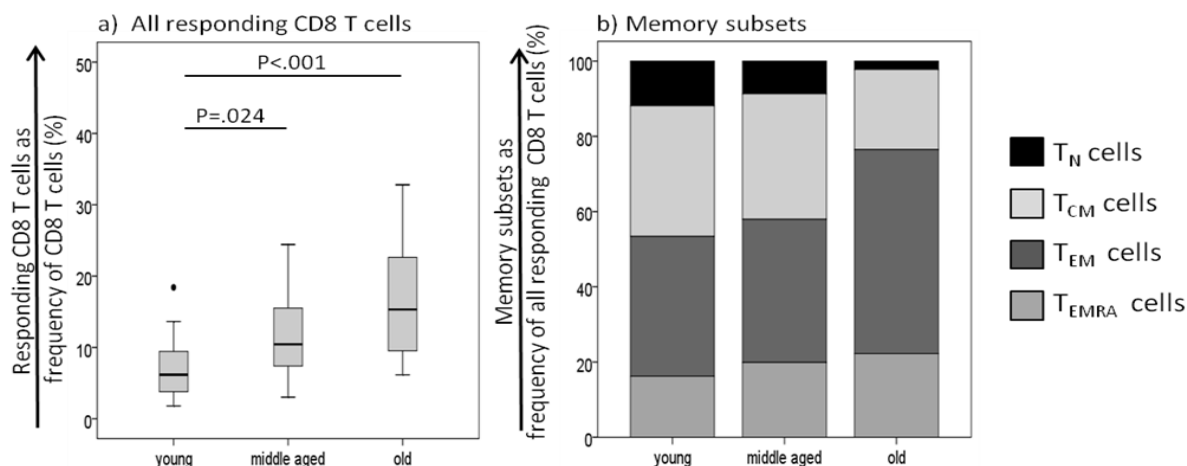


Figure 31: **Frequencies and memory subsets of responding CD8 T cells in three age groups after OKT3 stimulation.** a) Box plots represent the frequencies of responding CD8 T cells and b) stacked bars display mean frequencies of the four memory subsets in responding CD8 T cells after stimulation with OKT3 in young (n=27), middle-aged (n=11) and old (n=12) donors.

In summary, the T cell response size to pp65 and IE1 increased proportionally and absolutely with advancing age. Additionally, the memory subset distribution of pp65 and IE1-specific T cells changed towards a more differentiated phenotype with age and with increasing response size to pp65. The memory subset distribution of the entire T cell population became progressively more differentiated, as the response size to pp65 increased. The frequency and memory T cell subsets of the CD4 T cell response to PPD or OKT3 stimulation did not change with advancing age of the donor. The CD8 T cell response upon OKT3 stimulation increased with advancing age and contained proportionally more T_{EM} and T_{EMRA} cells in older people compared to young people.

6.4.2 Polyfunctional T cell responses

6.4.2.1 Functional heterogeneity of T cell responses

In this chapter, the functional composition of CMV-specific T cells is analysed in detail. Four functional read-outs were combined to increase sensitivity: CD40L, IFN γ , IL2, and TNF. Although these markers identify the majority of specific T cells, this is unlikely to be an inclusive population since other activation markers and dysfunctional cells were not measured. Rather than representing all possible 15 combinations of the four markers (2^4 minus 1 for the non-responsive subset), subpopulations were combined according to the number of expressed

activation markers. For example, all cells expressing one of the four activation markers only (IFN γ , IL2, CD40L or TNF) are grouped as T cells producing one activation marker. Subsets with more than one marker were considered polyfunctional for this purpose (this may differ from some published reports as there is no commonly accepted definition of the term).

Polyfunctional CD4 T cells increased with the pp65-specific T cell response size (Figure 32). The larger the T cell response size after stimulation with pp65, the greater the number of CD4 T cells that produced three or four of the analysed activation markers.

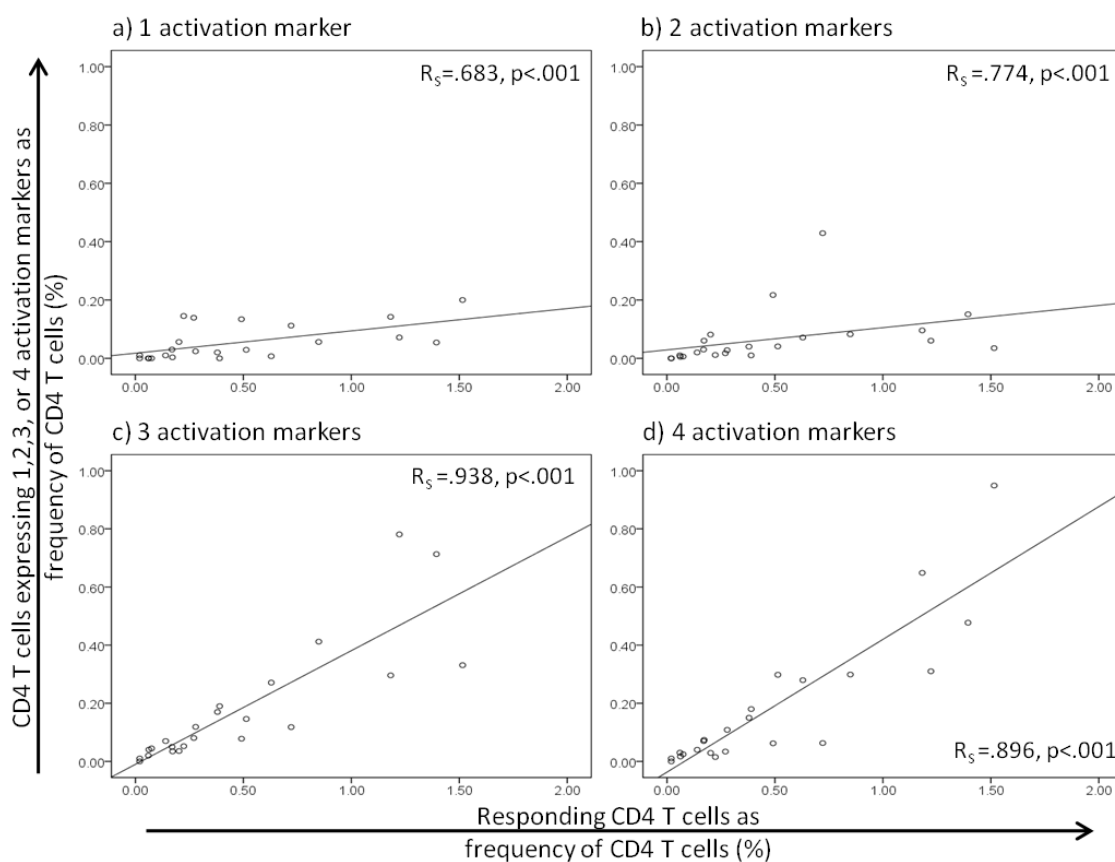
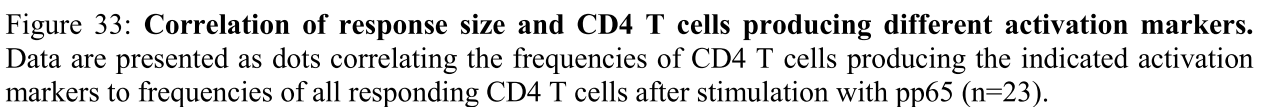


Figure 32: **Correlation of response size and polyfunctionality as frequency of all CD4 T cells.** Data are presented as dots correlating the frequencies of mono- and polyfunctional CD4 T cells to response size of pp65-specific CD4 T cells ($n=23$). Shown are cells producing a) one, b) two, c) three or d) four of the following activation markers: CD40L, IFN γ , IL2 and TNF.

Virtually all CD4 T cells expressing three activation markers produced CD40L, IFN γ and TNF simultaneously but no IL2 (Figure 33). Most of the pp65-specific CD4 T cells in this study expressed CD40L, IFN γ and TNF simultaneously with or without IL2 (Figure 33 and supplemental Figure 65).



In contrast to CD4 T cells, the increased number of CD8 T cells that responded to IE1 and pp65 stimulation was mainly due to a larger number of CD8 T cells that produced only two of the four analysed markers (Figure 34 and Figure 36). Virtually all these cells expressed IFN γ and TNF but not CD40L or IL2 (Figure 35, Figure 37 and supplemental Figure 66).

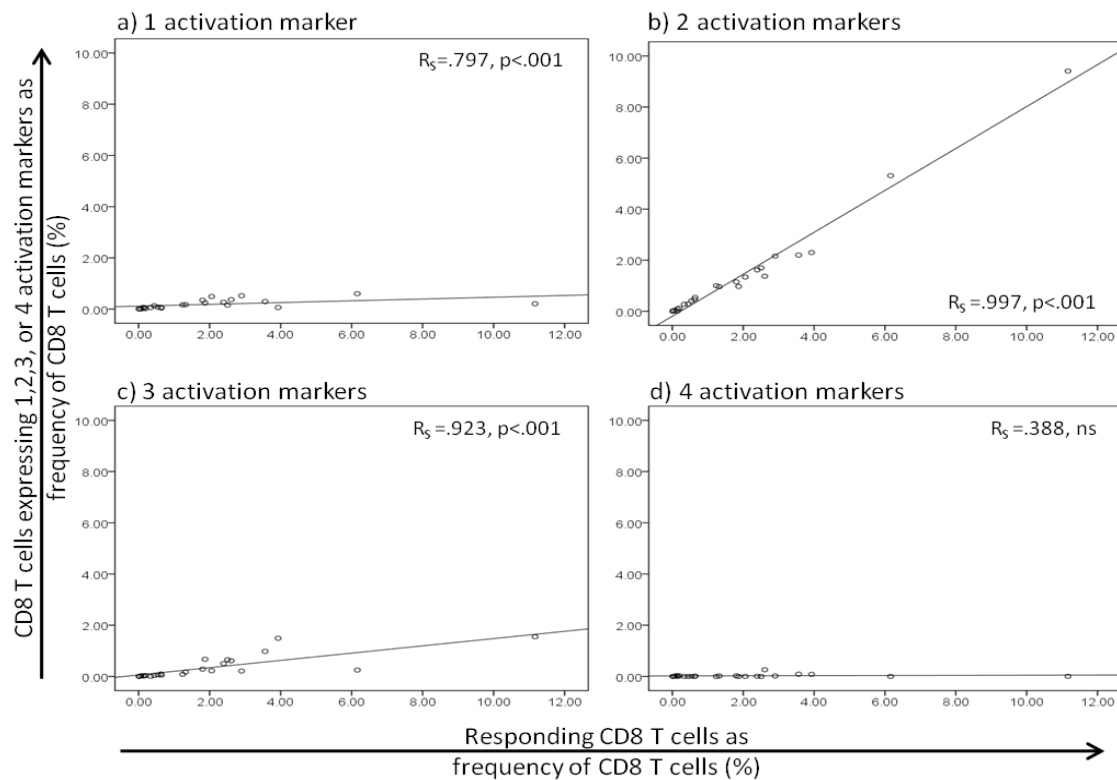


Figure 34: **Correlation of response size and polyfunctionality in pp65-specific CD8 T cells.** Data are presented as dots correlating the frequencies of mono- and polyfunctional cells to frequencies of pp65-specific CD8 T cells (n=23). Shown are cells producing a) one, b) two, c) three and d) four of the following activation markers: CD40L, IFN γ , IL2 and TNF.

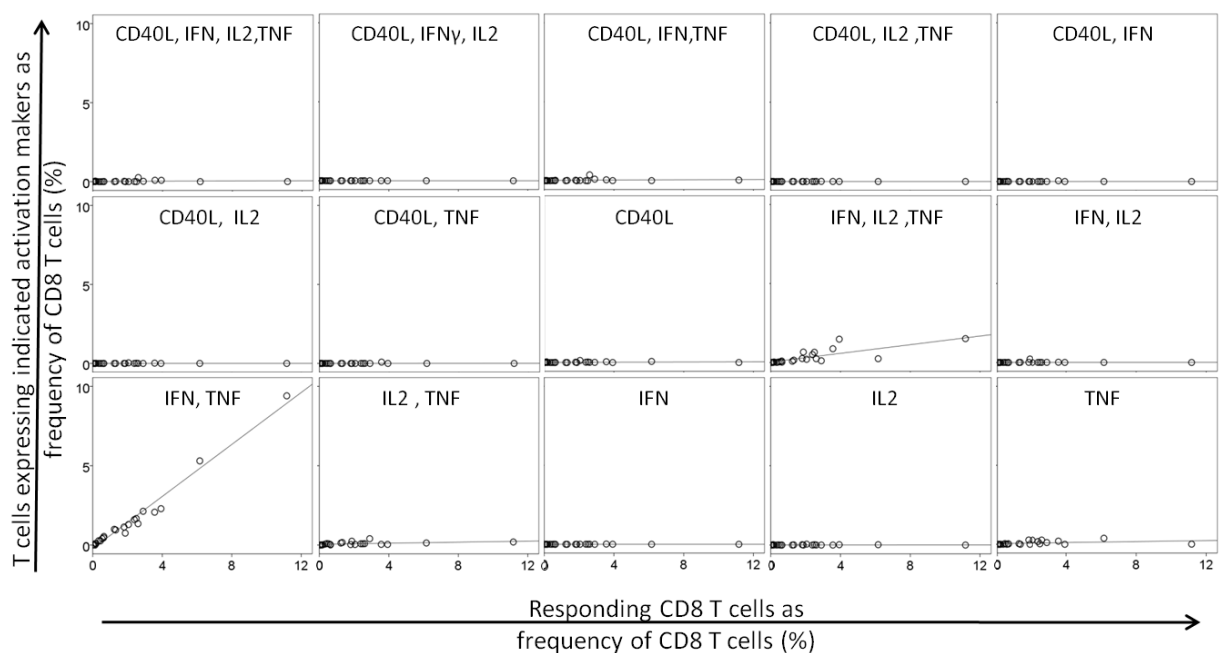


Figure 35: **Correlation of response size and cells producing different activation markers in pp65-specific CD8 T cells.** Data are presented as dots correlating the frequencies of CD8 T cells producing the indicated activation markers to frequencies of all pp65-specific CD8 T cells (n=23).

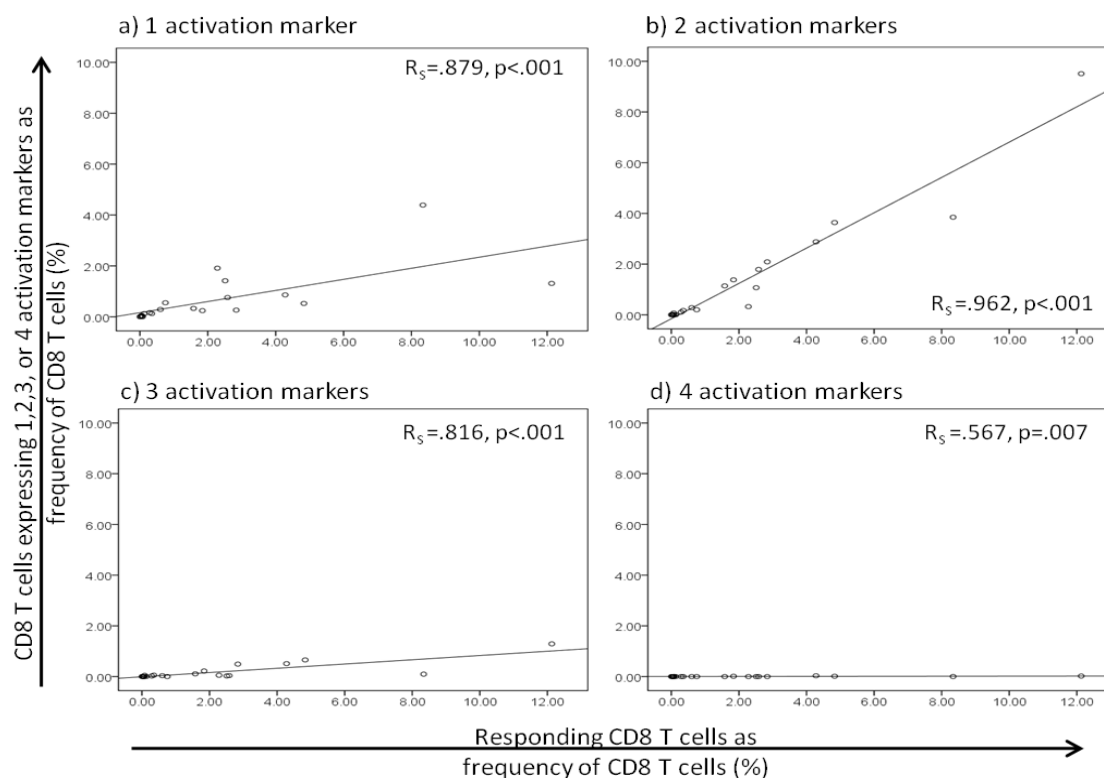


Figure 36: **Correlation of response size and polyfunctionality in IE1-specific CD8 T cells.** Data are presented as dots correlating the frequencies of mono- and polyfunctional cells to frequencies of IE1-specific CD8 T cells (n=21). Shown are cells producing a) one, b) two, c) three and d) four of the following activation markers: CD40L, IFN γ , IL2 and TNF.

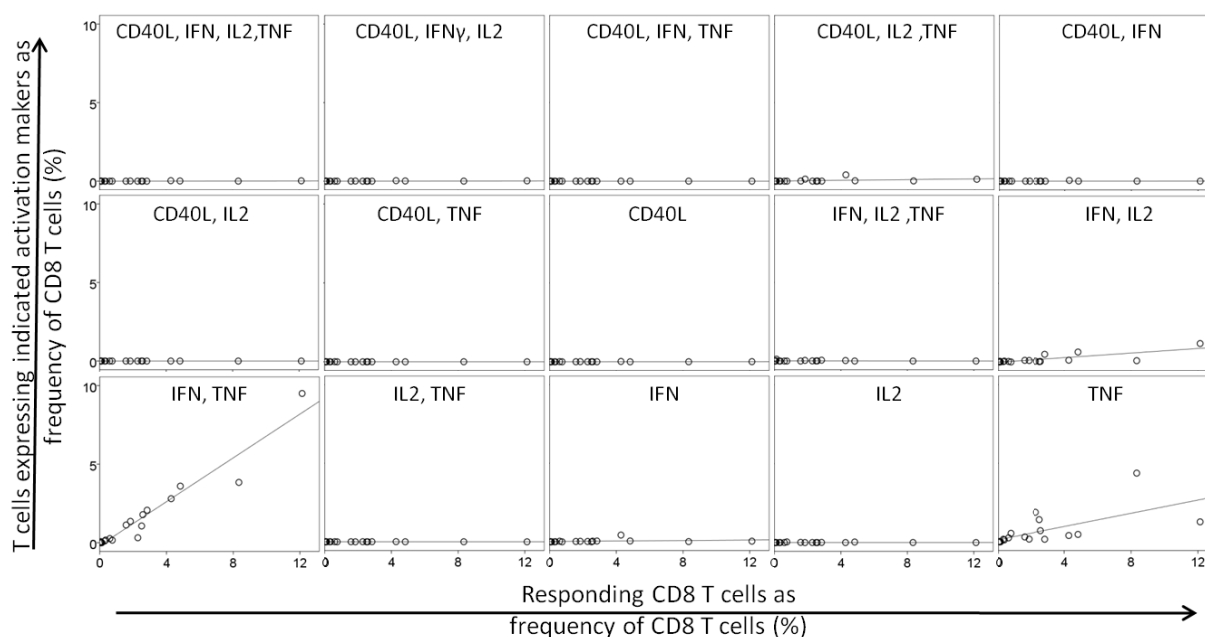


Figure 37: **Correlation of response size and cells producing different activation markers in IE1-specific CD8 T cells.** Data are presented as dots correlating the frequencies of CD8 T cells producing the indicated activation markers to frequencies of all IE1-specific CD8 T cells (n=21).

The frequency of polyfunctional T cells increased with expanding T cell response size to stimulation with pp65 or IE1. Responding CD4 T cells mainly produced three or four of the analysed activation markers (CD40L, IFN γ , TNF with or without IL2), whereas CD8 T cells predominantly produced two of the analysed activation markers (IFN γ and TNF) following stimulation with pp65 or IE1.

6.4.2.2 Memory subset distribution of T cell responses

It was of interest to investigate the polyfunctionality of CMV-specific T cells with regards to the memory T cell subset distribution.

Following stimulation with pp65 most activated CD4 T cells were observed in the CD4 T_{EM} cell subset (Figure 38). About 90% of pp65-specific CD4 T_{CM} and T_{EM} cells were polyfunctional, whereas just 60% of T_{EMRA} cells were polyfunctional, with most T cells producing three or four activation markers. Only a few CD4 T_N cells were activated after stimulation with pp65 and these cells were mainly monofunctional. Only few T_{EMRA} cells became activated and about 50% of these were polyfunctional T cells.

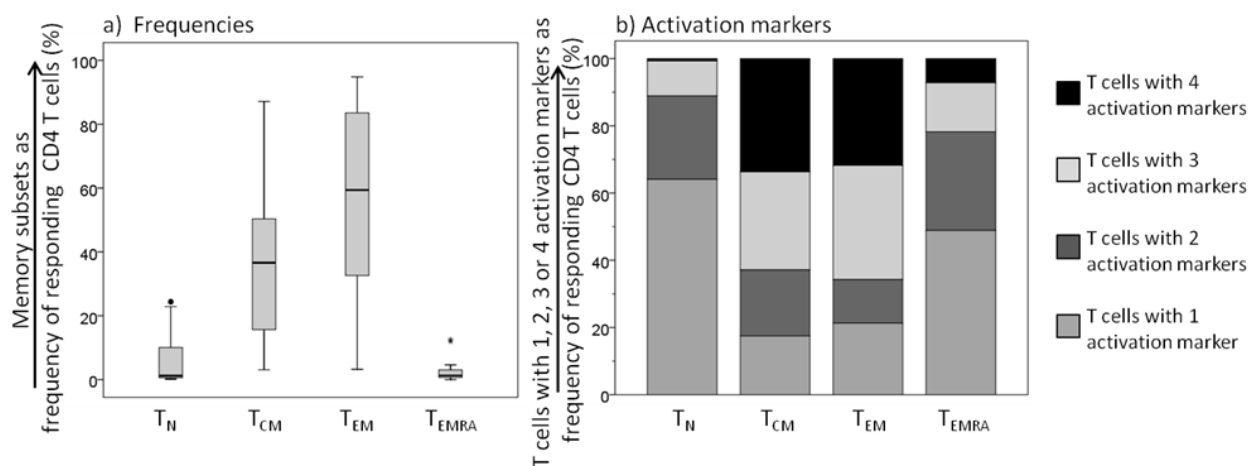


Figure 38: **pp65-specific CD4 T cell subsets in the four memory subsets.** a) Box plots display the frequencies of responding CD4 T cells in the four memory subsets and b) stacked bars show the mean frequencies of CD4 T cells expressing 1, 2, 3 and 4 activation markers simultaneously after stimulation with pp65 (n=22).

Most pp65 and IE1-specific CD8 T cells were found to belong to the T_{EM} cell subset (Figure 39 and Figure 40). The pp65-specific CD8 T cells were mainly polyfunctional with 80-90% of T_{CM}, T_{EM} and T_{EMRA} cells expressing more than 1 activation marker. IE1-specific CD8 T cells

displayed more monofunctional cells, with 70% of T_{CM} and just 45% of T_{EM} and T_{EMRA} cells being polyfunctional. Of these polyfunctional T cells, most displayed two activation markers.

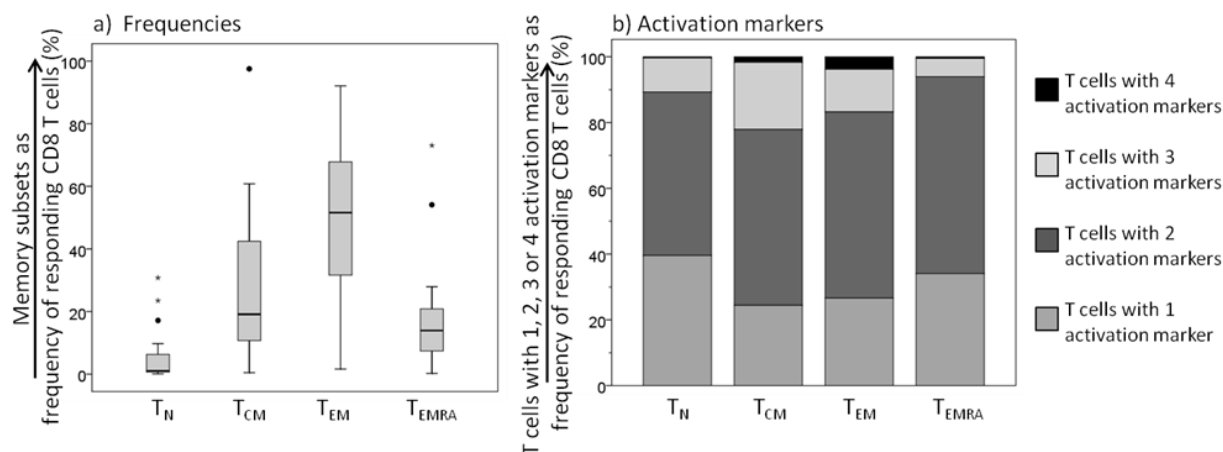


Figure 39: **pp65-specific CD8 T cell subsets in the four memory subsets.** a) Box plots display the frequencies of responding CD8 T cells in the four memory subsets and b) stacked bars show the mean frequencies of CD8 T cells expressing 1, 2, 3 and 4 activation markers simultaneously after stimulation with pp65 (n=22).

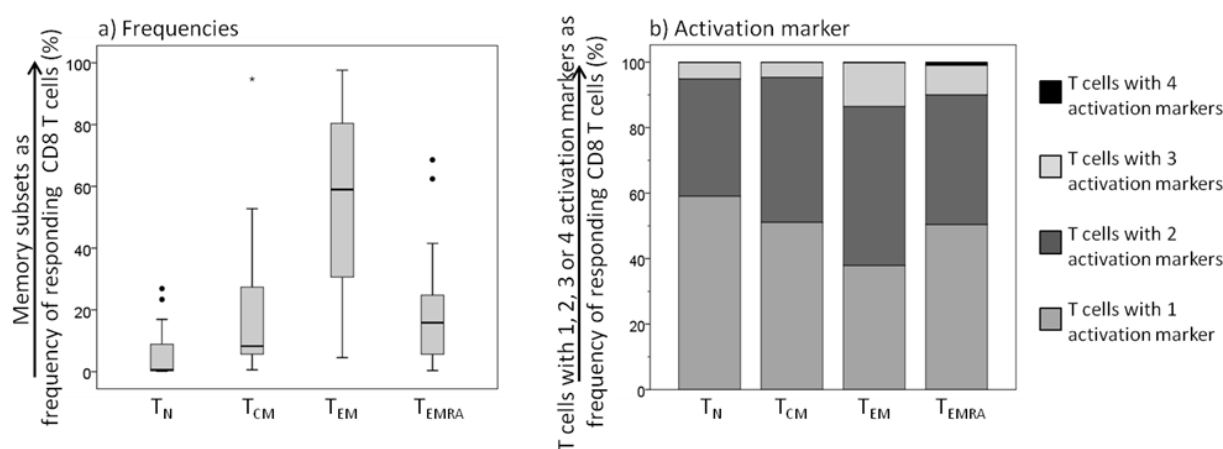


Figure 40: **IE1-specific CD8 T cell subsets in the four memory subsets.** a) Box plots display the frequencies of responding CD8 T cells in the four memory subsets and b) stacked bars show the mean frequencies of CD8 T cells expressing 1, 2, 3 and 4 activation markers simultaneously after stimulation with IE1 (n=21).

PPD was used as recall antigen in order to analyse whether the memory subset distribution of the antigen-specific T cell response to CMV proteins was representative for other antigens. In contrast to pp65-specific CD4 T cells, the memory distribution of PPD-specific CD4 T cells consisted mainly of T_{CM} cells (Figure 41). 70% of PPD-specific CD4 T_{CM} cells and 80% of PPD-specific T_{EM} cells were polyfunctional, most of them expressing three or four of the analysed

activation markers following PPD stimulation. The few activated CD4 T_N and T_{EMRA} cells were mainly monofunctional.

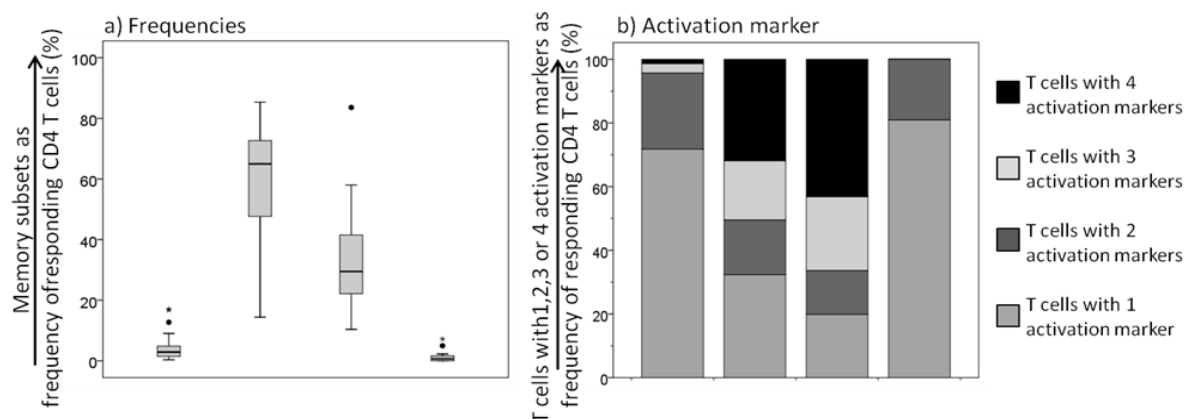


Figure 41: **PPD-specific CD4 T cell subsets in the four memory subsets.** a) Box plots display the frequencies of responding CD4 T cells in the four memory subsets and b) stacked bars show the mean frequencies of CD4 T cells expressing 1, 2, 3 and 4 activation markers simultaneously after stimulation with PPD (n=24).

Additionally polyclonally activated T cells were also analysed. Most of the CD4 T cells that responded to OKT3 were present in the T_{CM} cell subset (Figure 42). Analysis of polyclonally stimulated CD4 T cells showed that only 30% of T_{CM} , but 55% of T_{EM} and T_{EMRA} cells were producing more than one of the analysed activation markers. Further differentiated CD4 T cells expressed proportionally more activation markers simultaneously.

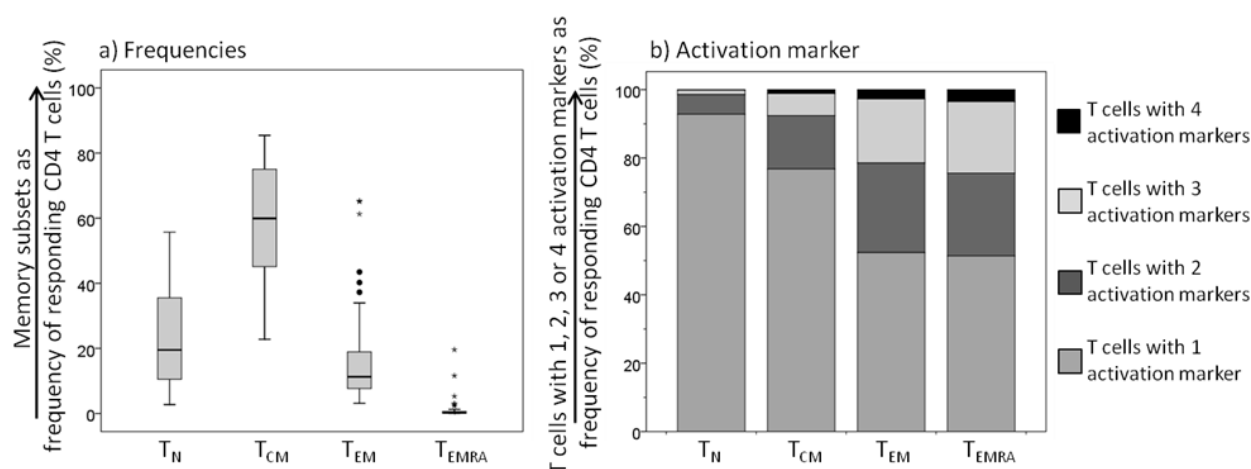


Figure 42: **CD4 T cell after polyclonal stimulation subsets in the four memory subsets.** a) Box plots display the frequencies of responding CD4 T cells in the four memory subsets and b) stacked bars show the mean frequencies of responding CD4 T cells expressing 1, 2, 3 and 4 activation markers simultaneously after stimulation with OKT3 (n=50).

The majority of CD8 T cells that responded following OKT3 stimulation were equally distributed between the CD8 T_{CM} and T_{EM} cell compartments (Figure 43). As observed for CD4 T cells, responding CD8 T_N cells were predominantly monofunctional following polyclonal stimulation. Although further differentiated CD8 T cells expressed proportionally more activation markers simultaneously (40% of T_{CM} and 50% of T_{EM} and T_{EMRA} cells), only very few T cells expressed three or four of the analysed activation markers.

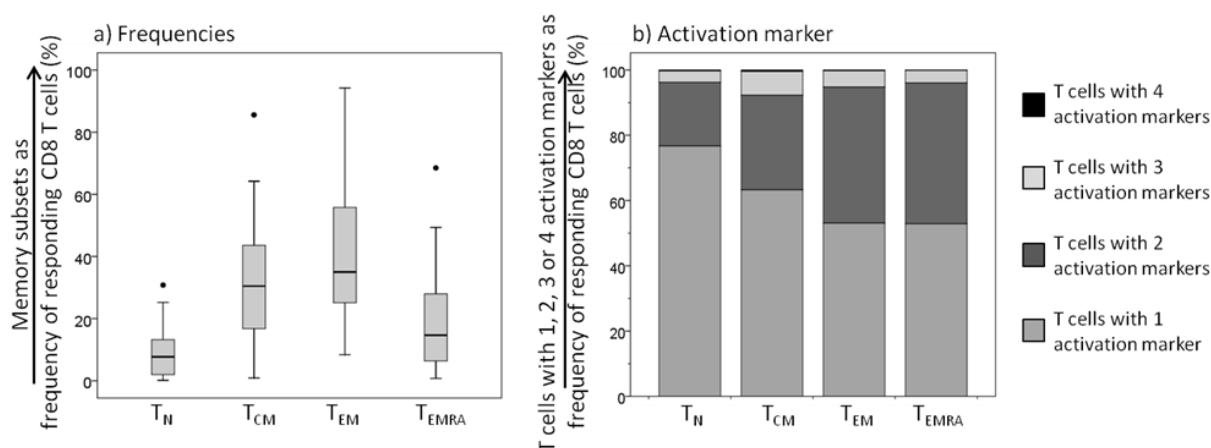


Figure 43: **CD8 T cells after polyclonal stimulation in the four memory subsets.** a) Box plots display the frequencies of responding CD4 T cells in the four memory subsets and b) stacked bars show the mean frequencies of CD8 T cells expressing 1, 2, 3 and 4 activation markers simultaneously after stimulation with OKT3 (n=50).

Depending on the stimulus used, activated T cells were mainly seen in the T_{CM} cell subset (polyclonally stimulated and PPD-specific CD4 T cells) and T_{EM} cell subset (polyclonally stimulated CD8 T cells and CMV-specific CD4 and CD8 T cells). Although T_{EM} cells were slightly more polyfunctional than T_{CM}, the polyfunctionality of memory T cells appeared to be mainly influenced by the stimulus used and not by the memory T cell subset.

6.4.2.3 Qualitative differences of T cell responses

CMV-specific T cells were also analysed for quantitative differences in the production of activation markers. The standardised MFI could only be calculated if all mono- and polyfunctional subpopulation were present in the same donors. Therefore the numbers of donors differ from the other analyses. After stimulation with pp65, CD4 T cells that produced more activation markers simultaneously also had increased MFIs for IFN γ , TNF and CD40L per cell (Figure 44). The MFIs for IFN γ , TNF and CD40L was lowest in T cells displaying just one activation marker and was highest in cells displaying all four activation markers. The difference

in the MFIs between cells producing only one or all four cytokines was significant for all activation markers. The largest difference was seen for TNF (10-fold increase, $p < 0.001$), followed by IFN γ (6-fold increase, $p < 0.001$), CD40L (4-fold increase, $p < 0.001$) and finally IL2 (less than 2-fold increase, $p = 0.045$). The highest MFI for IL2 from CD4 T cells was seen when IL2 was produced with one other activation marker. Half the donors (10 out of 21) had CD4 T cells that produced IL2 and one other marker only. In the majority of cases, IL2 was produced in conjunction with CD40L (data not shown). The correlation between the quality and quantity of activation markers was calculated with Spearman's correlation R_s (Table 12).

The same pattern for all activation markers was observed in CD4 T cells stimulated with OKT3 or PPD (Table 12, supplemental Figure 67 and Figure 68).

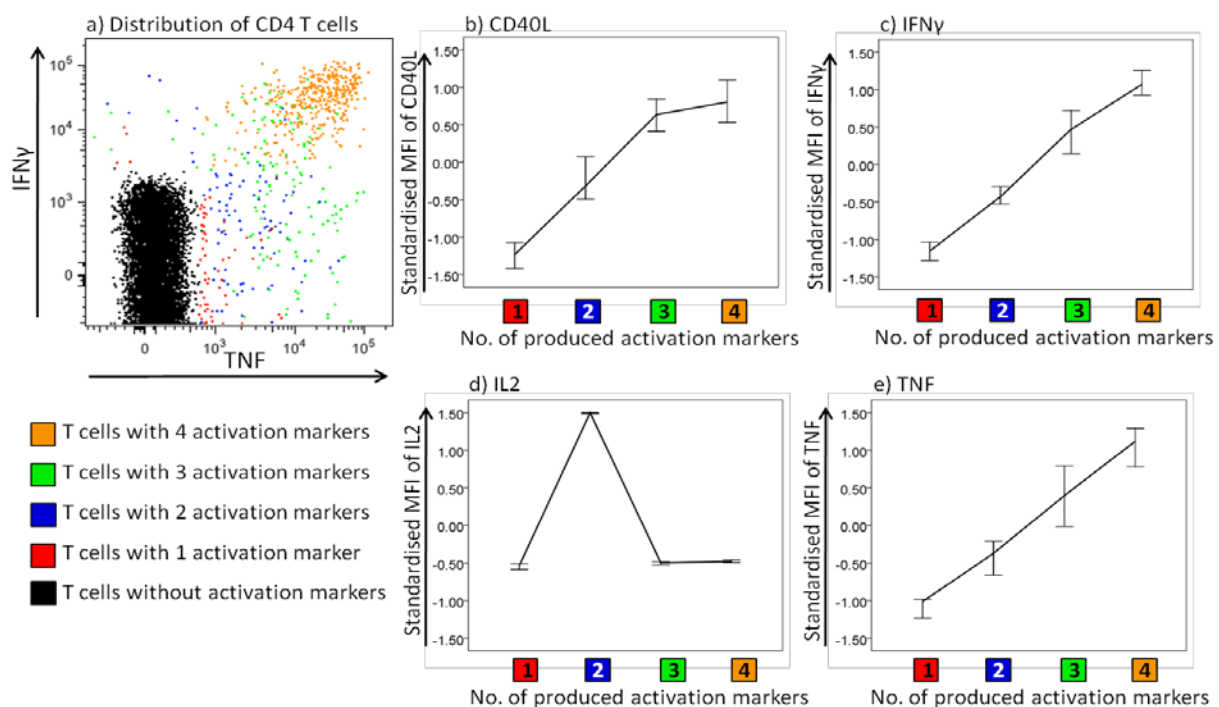


Figure 44: **Expression of activation markers in pp65-specific CD4 T cells.** Representative dot plot shows the distribution of CD4 T cells after stimulation with pp65 for 16 hours. Line curves show standardised MFIs of a) IFN γ , b) IL2, c) TNF and d) CD40L in CD4 T cells expressing one (red), two (blue), three (green) or four (orange) activation markers (n=20).

	CD40L	IFN γ	IL2	TNF
pp65-stimulated CD4 T cells	$R_s = 0.864$, $p < 0.001$	$R_s = 0.954$, $p < 0.001$	$R_s = 0.148$, ns	$R_s = 0.893$, $p < 0.001$
pp65-stimulated CD8 T cells	$R_s = 0.889$, $p < 0.001$	$R_s = 0.885$, $p < 0.001$	$R_s = 0.096$, ns	$R_s = 0.870$, $p < 0.001$
OKT3-stimulated CD4 T cells	$R_s = 0.907$, $p < 0.001$	$R_s = 0.939$, $p < 0.001$	$R_s = 0.238$, $p = 0.012$	$R_s = 0.959$, $p < 0.001$
OKT3-stimulated CD8 T cells	$R_s = 0.777$, $p < 0.001$	$R_s = 0.897$, $p < 0.001$	$R_s = 0.116$, ns	$R_s = 0.952$, $p < 0.001$
IE1-stimulated CD8 T cells	$R_s = 0.893$, $p < 0.001$	$R_s = 0.789$, $p < 0.001$	$R_s = 0.143$, ns	$R_s = 0.761$, $p < 0.001$
PPD-stimulated CD4 T cells	$R_s = 0.948$, $p < 0.001$	$R_s = 0.815$, $p < 0.001$	$R_s = 0.285$, $p = 0.02$	$R_s = 0.962$, $p < 0.001$

Table 12: Correlations of amount of indicated activation marker per cell and number of displayed activation markers. Shown are Spearman's correlation coefficient R_s and p-value.

Similar to pp65-specific CD4 T cells, pp65-specific CD8 T cells that produced several activation markers had increased MFIs for IFN γ , TNF and CD40L per cell (Figure 45). Although CD8 T cells producing all four activation markers, including CD40L were very rare, these cells contained the highest amounts of IFN γ , TNF and CD40L. The MFI for IFN γ , TNF and CD40L increased from T cells producing just one activation marker to all four. CD8 T cells producing IL2 had the highest MFI for IL2 when producing only 2 cytokines. In CD8 T cells all three combinations of cells producing IL2 and CD40L or IL2 and IFN γ or IL2 and TNF were observed (data not shown). All three subsets formed less than 5% of all responding CD8 T cells in all donors. The difference in the MFI between pp65-specific CD8 T cells producing only one to all four cytokines was very similar to the differences in CD4 T cells. Again, this was significant for all activation markers. The largest difference was seen for TNF (11-fold increase, $p < 0.001$), followed by IFN γ (5-fold increase, $p < 0.001$), CD40L (4-fold increase, $p < 0.001$) and the smallest difference in the MFI was seen for IL2 (less than 2-fold increase, $p < 0.001$).

The same pattern of activation markers was observed in CD8 T cells stimulated with either IE1 or OKT3 (Table 12, supplemental Figure 69 and Figure 70).

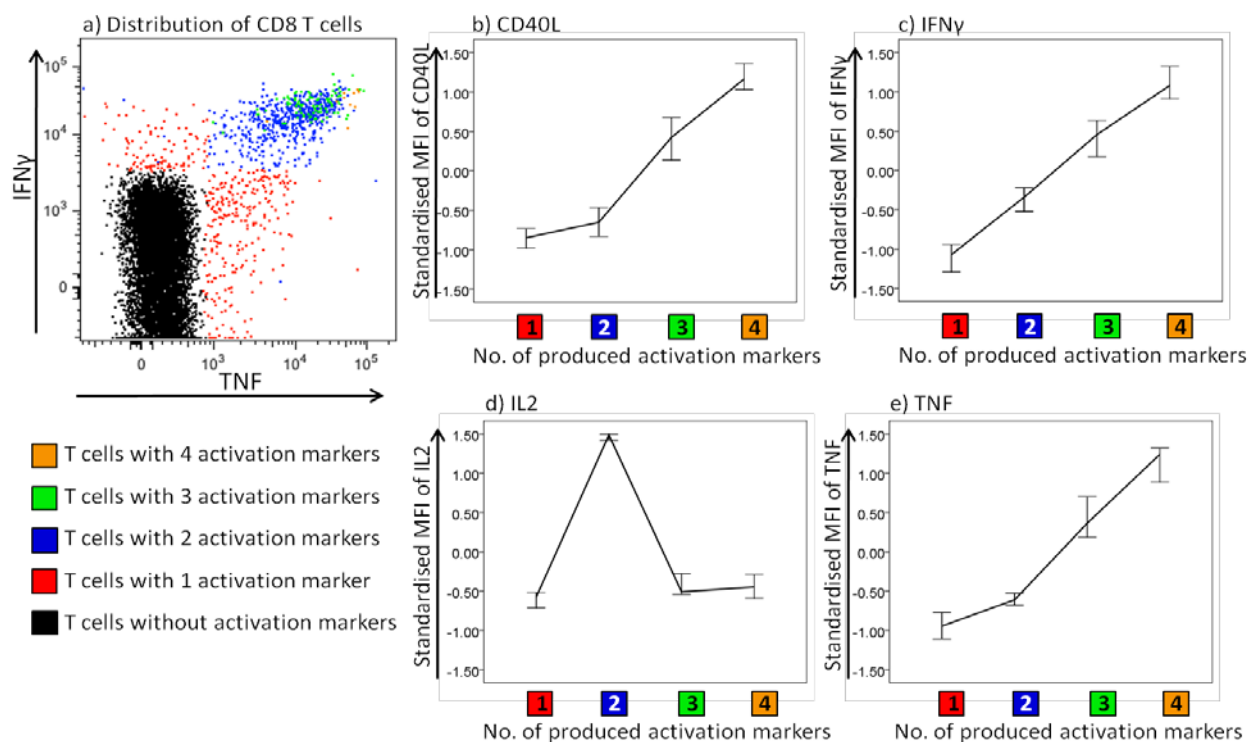


Figure 45: **Expression of activation markers in pp65-specific CD8 T cells.** Representative dot plot shows the distribution of pp65-specific CD8 T cells after stimulation with pp65. Line curves show standardised MFIs of a) IFN γ , b) IL2, c) TNF and d) CD40L in CD8 T cells expressing one (red), two (blue), three (green) or four (orange) activation markers (n=20).

In conjunction with the increased expression of activation markers, a downregulation of surface CD3 and CD4 was seen on cytokine-producing pp65-specific CD4 T cells (Figure 46, Table 1). The downregulation of CD3 and CD4 was positively correlated with the number of activation markers the T cell produced. CD4 T cells producing three or four activation markers had the least amount of CD3 and CD4 molecules on the surface.

Downregulation of CD3 and CD8 was also seen on pp65-specific CD8 T cells and was enhanced the more cytokines the cell was producing (Figure 46). CD8 T cells producing three or four cytokines had the least amount of CD3 and CD8 molecules on the surface.

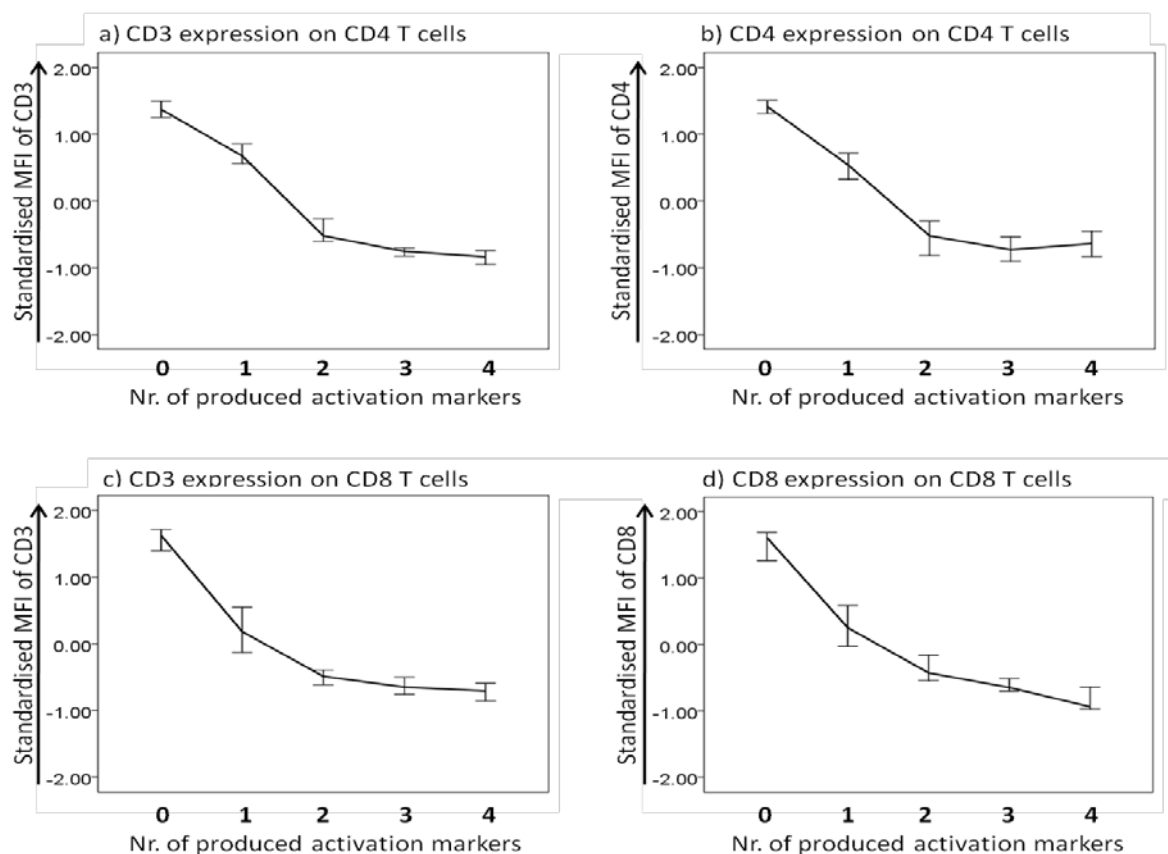


Figure 46: **Downregulation of CD3, CD4 and CD8 on T cells after pp65-specific stimulation.** Line curves represent standardised MFIs of a) CD3, b) CD4 expression on pp65-specific CD4 T cells and c) CD3, d) CD8 expression on pp65-specific CD8 T cells expressing different numbers of markers (n=20).

Downregulation of CD3 and the respective co-receptor was also observed on IE1-specific CD8 T cells, on PPD-specific CD4 T cells and on polyclonally stimulated CD4 and CD8 T cells (Table 13, supplemental Figure 72, Figure 71 and Figure 73).

	CD3 on CD4 T cells	CD4 on CD T cells	CD3 on CD8 T cells	CD8 on CD8 T cells
pp65-stimulated T cells	$R_s = 0.927$, $p < 0.001$	$R_s = 0.787$, $p < 0.001$	$R_s = 0.767$, $p < 0.001$	$R_s = 0.842$, $p < 0.001$
OKT3-stimulated T cells	$R_s = 0.887$, $p < 0.001$	$R_s = 0.450$, $p < 0.001$	$R_s = 0.602$, $p < 0.001$	$R_s = 0.625$, $p < 0.001$
IE1-stimulated T cells			$R_s = 0.782$, $p < 0.01$	$R_s = 0.625$, $p < 0.001$
PPD-stimulated T cells	$R_s = 0.968$, $p < 0.001$	$R_s = 0.886$, $p < 0.001$		

Table 13: Correlations of CD3 and co-receptor downregulation and number of displayed activation markers. Shown are Spearman's correlation coefficient R_s and p-value.

These data suggest that polyfunctional CD4 T cells were, independent of the used stimulation, more efficient than monofunctional cells because they produced several effector molecules in parallel and synthesised more of each cytokine on a per cell level [124-129]. At the same time, the downregulation of the surface markers CD3, CD4 and CD8 were stronger on polyfunctional cells than on monofunctional cells indicating a stronger activation.

6.5 Effect of CMV infection on unrelated T cell responses

It is generally accepted that CMV infection alters the immune system in many different ways. To investigate if CMV infection had an effect on polyclonally stimulated T cells, PBMC from CMV-seropositive (n=24) and CMV-seronegative donors (n=25) were stimulated with OKT3 and analysed.

There were only minor differences in the response size of the CD4 T cell compartment after OKT3 stimulation in CMV-seronegative and CMV-seropositive donors. However, the distribution of memory subsets was significantly altered (Figure 47). Donors with CMV infection had proportionally more responding CD4 T_{EM} and T_{EMRA} cells (p<0.001 and p=0.001 respectively) compared to CMV-negative donors, while T_{CM} cells were reduced (p=0.048). In terms of absolute numbers, CMV infected donors had significantly more responding CD4 T_{EM} and T_{EMRA} cells (supplemental Figure 74, p<0.001 for both). Polyclonally activated CD4 T cells from CMV-positive donors contained proportionally more polyfunctional and less monofunctional T cells than CD4 T cells from CMV-negative donors (Figure 47, p=0.005). Activated CD4 T cells from CMV-infected and uninfected donors produced the same quantities of cytokine per cell (data not shown).

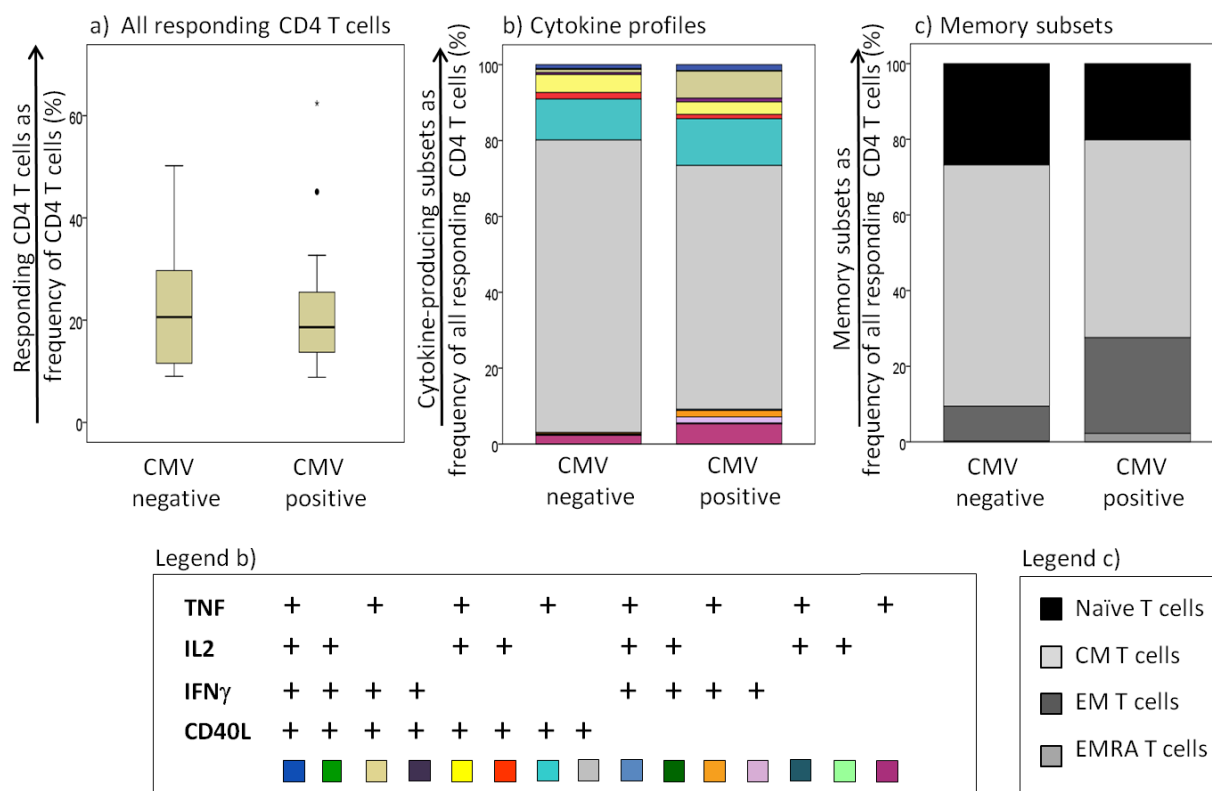


Figure 47: **Frequencies, cytokine profiles and memory subsets of responding CD4 T cells following polyclonal stimulation in CMV-seronegative and CMV-seropositive donors.** a) Box plots represent the frequencies of responding CD4 T cells, b) stacked bars represent the cytokine profiles and c) stacked bars display frequencies of the four memory subsets in responding CD4 T cells after stimulation with OKT3 for CMV-negative (n=25) and CMV-positive donors (n=24).

CMV infected donors had twice the frequency of responding CD8 T cells after OKT3 stimulation compared to CMV-negative donors (Figure 47, median=13% and median 7% respectively, $p<0.001$). Analysis of the absolute numbers showed a greater than threefold increase of CD8 T cells producing cytokines after OKT3 stimulation (supplemental Figure 74, median = 74874 cells/ml blood and median= 28622 cells/ml blood respectively, $p<0.001$).

The correlation between the frequencies of pp65-specific CD8 T cells and polyclonally activated CD8 T cells was not significant but the same correlation in terms of absolute numbers was significant ($R_s = 0.48$, $p=0.018$, data not shown). The bigger the pp65-specific T cell response was, the bigger was the OKT3-induced CD8 T cell response. CMV infection did not only alter the numbers of responding T cells but also the memory T cell subset distribution. CMV-infected donors had, in proportion, significant fewer responding CD8 T_N and T_{CM} cells than donors without CMV infection (Figure 48, $p=0.013$ and $p=0.008$ respectively). The absolute numbers of

responding CD8 T cells after polyclonal stimulation contained more T_{EM} and T_{EMRA} cells in CMV-seropositive donors compared to seronegative donors (supplemental Figure 75, $p < 0.001$ for both subsets).

Polyclonally activated CD8 T cells from CMV-positive donors contained proportionally more polyfunctional and less monofunctional T cells as compared to CMV-negative donors (Figure 48, $p = 0.007$). No differences were found regarding the MFIs of the activation markers between CMV-positive and negative donors (data not shown).

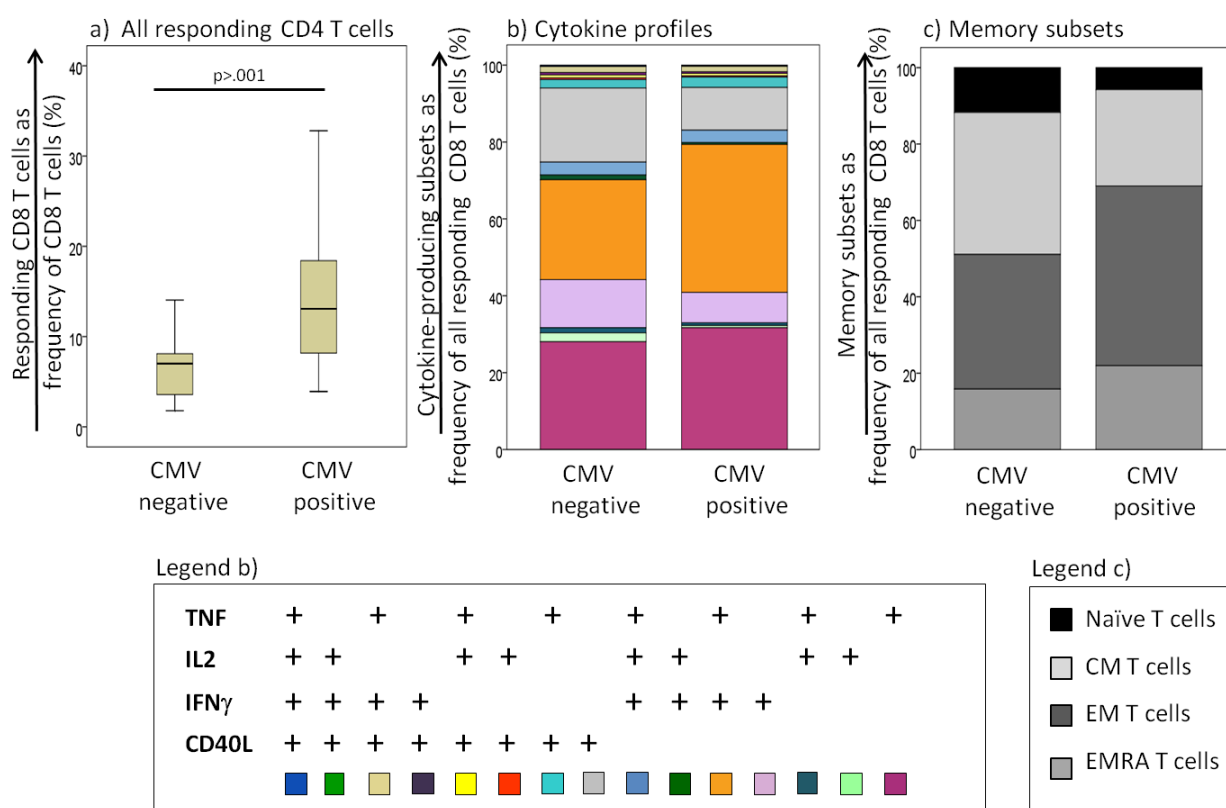


Figure 48: Frequencies and distribution of responding CD8 T cells in four memory subsets after OKT3 stimulation in CMV-seronegative and CMV-seropositive donors. a) Box plots represent the frequencies of responding CD8 T cells, b) stacked bars display the cytokine profiles and c) stacked bars display the four memory subsets in responding CD8 T cells after stimulation with OKT3 for CMV-negative ($n=25$) and CMV-positive donors ($n=24$).

The frequencies of responding CD8 T cells after OKT3 stimulation increased significantly with age regardless of the CMV-serostatus ($R_s = 0.480$, $p < 0.001$) although a stronger incline was observed in CMV-seropositive people (Figure 49). The same trend was seen in absolute numbers of CD8 T cells per ml blood, but the correlation of response size with age was only significant in

all donors and not in CMV-positive or CMV-negative donors separately (supplemental Figure 76).

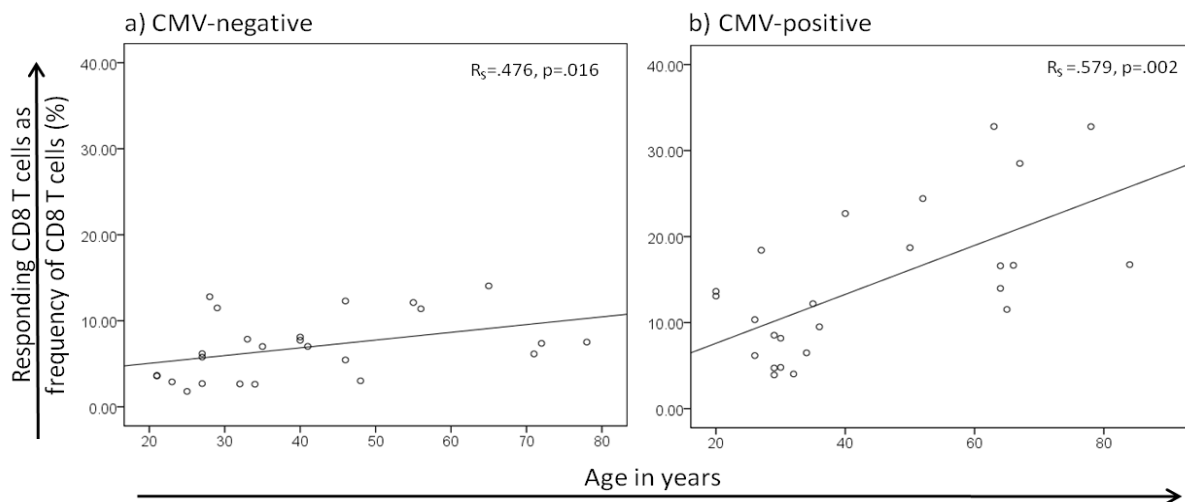


Figure 49: **Correlation of CMV status, age and response size after OKT3 stimulation.** Scatter plots represent the number of responding CD8 T cells after stimulation with OKT3 in a) CMV-negative (n=25) and b) CMV-positive (n=24) donors related to age of the donor.

CMV-infected (n=11) and uninfected (n=13) donors with a CD4 T cell response specific for PPD were analysed separately to investigate whether CMV infection influences other heterologous antigen-specific T cell responses as well.

Frequencies of PPD-specific CD4 T cells were similar in both groups, but donors with CMV infection had significantly higher absolute numbers of PPD-specific CD4 T cells than donors without CMV infection (Figure 50 and supplemental Figure 77, $p=0.018$). PPD-specific CD4 T cells contained, in proportion, significantly more CD4 T_{EM} and fewer T_{CM} cells in CMV-positive donors as compared to CMV-negative donors (Figure 50, $p<0.001$ and $p=0.003$ respectively). The absolute number of PPD-specific CD4 T_{EM} cells was higher in CMV-infected donors as compared to uninfected donors (supplemental Figure 77, $p=0.003$). The cytokine profiles of responding CD4 T cells in CMV-seropositive and negative people were very similar (Figure 50) and no differences were found regarding the MFIs of the activation markers between CMV-positive and negative donors (data not shown).

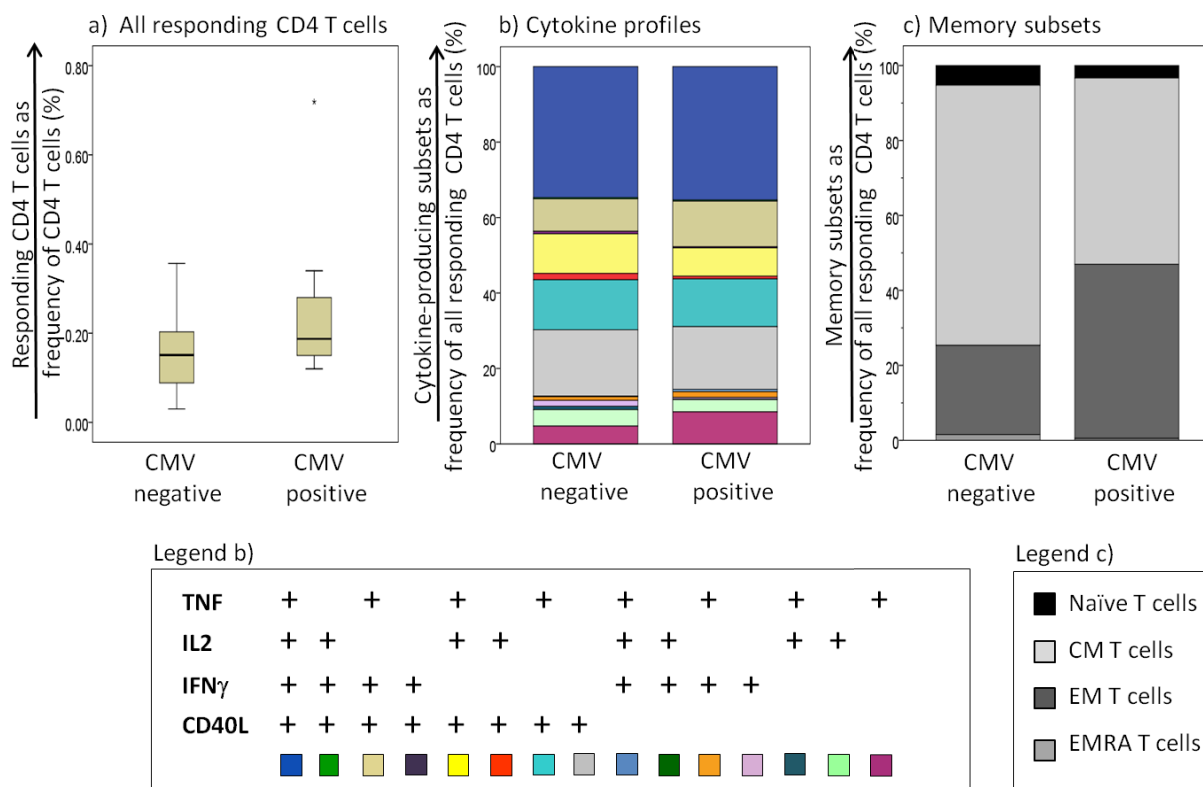


Figure 50: Frequencies, cytokine profiles and memory subset distribution of responding CD4 T cells following PPD stimulation in CMV-seronegative and CMV-seropositive donors. a) Box plots represent the frequencies of responding CD4 T cells, b) stacked bars display the cytokine profiles and c) stacked bars display the four memory subsets in responding CD4 T cells after stimulation with PPD for CMV-negative (n=13) and CMV-positive donors (n=11).

To summarise, CMV-infected donors had bigger PPD-specific CD4 T cell responses than CMV-seronegative donors. The T cell response consisted of more T_{EM} and T_{EMRA} cells in CMV-positive compared to CMV-negative people but no differences were found regarding the cytokine profile. CMV-infected donors also had bigger CD8 T cell responses to polyclonal stimulation than CMV-seronegative donors although the CD4 T cell response was not altered. The polyclonally stimulated T cell response consisted of more T_{EM} and T_{EMRA} cells and more polyfunctional T cells in CMV-positive than in CMV-negative people.

6.6 Effects of immunosuppressive drugs on CMV-specific T cell response

In order to investigate the effect of immunosuppressive drugs on polyfunctional CMV-specific T cells, samples were pre-incubated *in vitro* with either cyclosporin A (CSA) or tacrolimus

(FK506) overnight prior to stimulation with pp65 for 6 h. Each immunosuppressive drug was tested in three different concentrations (physiological level, fivefold less and fivefold greater concentration), in duplicates, on PBMC from three donors. PBMC were stained for IFN γ , IL2, CD40L, TNF and CD107a. CD107a is an established marker for degranulation [32].

All the following analyses were only carried out for CD8 T cells as the frequencies of pp65-specific CD4 T cells were very small (< 0.1% of all CD4 T cells) in the three donors. The frequencies of pp65-specific CD8 T cells were 0.39%, 1.02% and 1.32% of all CD8 T cells in the three donors.

Pre-incubation with either CSA or FK506 *in vitro* did not alter the frequencies of responding T cells after pp65 stimulation (Figure 51). Looking at the donors individually, one donor had slightly elevated levels of responding T cells, whereas the second donor had slightly reduced numbers and the third donor showed no differences in responding T cell frequencies after pre-incubation with immunosuppressive reagents.

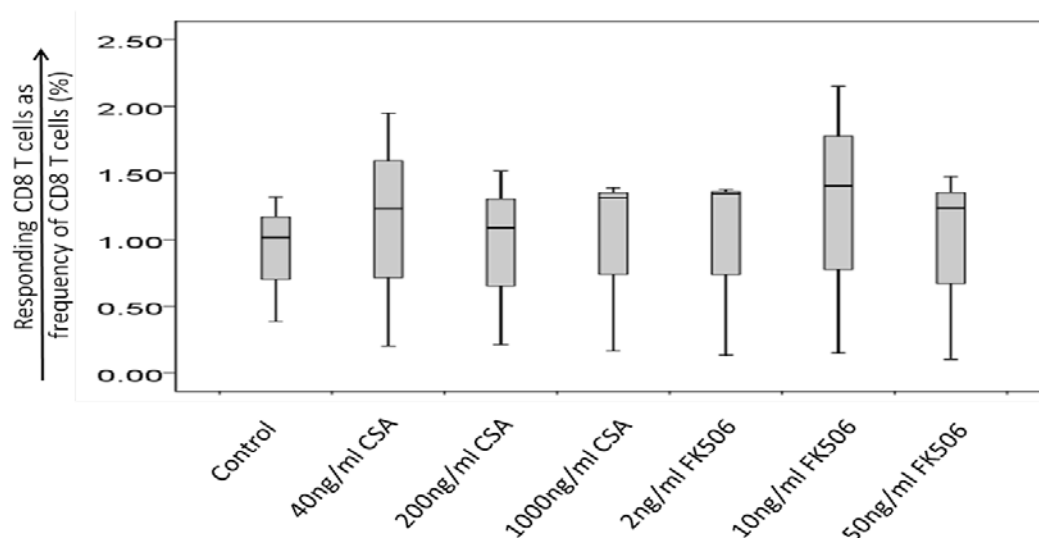


Figure 51: Frequencies of responding CD8 T cells after incubation with immunosuppressive and pp65 stimulation *in vitro*. Box plots show the frequencies of all responding CD8 T cells after pre-incubation with three different concentrations of either CSA (40ng/ml, 200ng/ml and 1000ng/ml) or FK506 (2 ng/ml, 10 ng/ml and 50ng/ml) and stimulation with pp65. The control cells were stimulated with pp65 but not treated with immunosuppressive drugs. All samples were done in duplicates (n=3).

Although there were no differences in the frequencies of responding CD8 T cells, the cytokine profile of the responding CD8 T cells was altered (Figure 52). Under the short term effect of

immunosuppressive drugs *in vitro*, all polyfunctional subsets decreased, whereas the subsets displaying only one function increased dose-dependently.

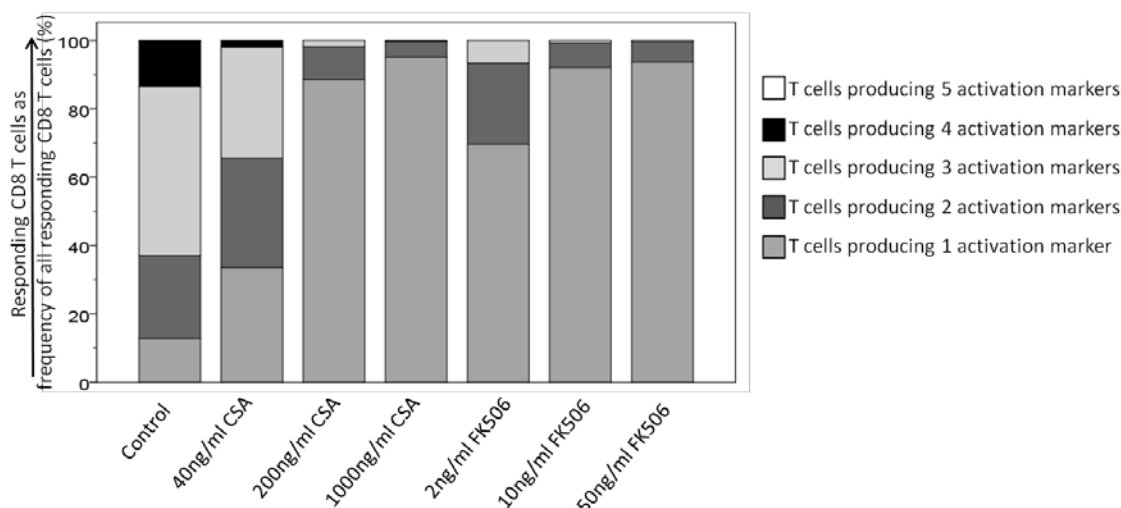


Figure 52: **Cytokine profile of pp65-specific CD8 T cells after incubation with immunosuppressive drugs and pp65 stimulation *in vitro*.** Stacked bars show the frequencies of CD8 T cells producing different numbers of activation marker simultaneously after pre-incubation with three different concentrations of either CSA (40ng/ml, 200ng/ml and 1000ng/ml) or FK506 (2 ng/ml, 10 ng/ml and 50ng/ml) and stimulation with pp65. Activation markers include CD40L, CD107a, IFN γ , IL2 and TNF. All samples were done in duplicates (n=3).

CD8 T cells stimulated with pp65 without immunosuppressive reagents mainly expressed CD107a, IFN γ and TNF- α . CD40L was expressed on very few cells (less than 0.03% of all CD8 T cells) and therefore not analysed in detail. Analysis of the cytokine profile revealed that the main function retained was the upregulation of CD107a, showing that the cells were degranulating but not expressing CD40L, IFN γ , IL2 or TNF (Figure 53, Figure 54 and Figure 55). The frequencies of T cells producing the cytokines IFN γ , IL2 and TNF were diminished in a dose-dependent manner by CSA and FK506. The cytokine profiles of PBMC incubated with either CSA or FK506 at physiological concentrations were very similar.

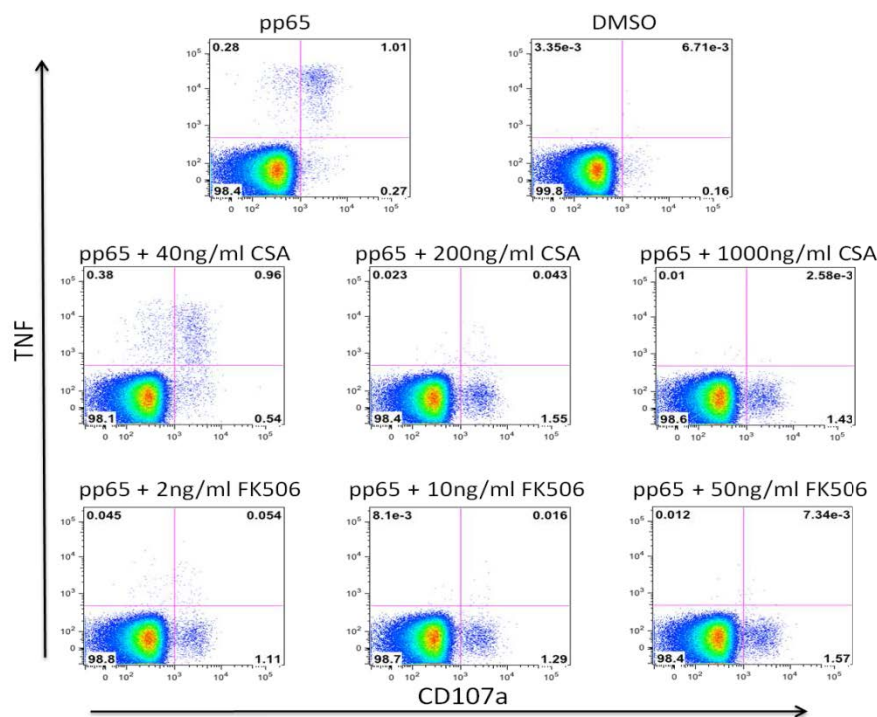


Figure 53: **CD8 T cells after incubation with immunosuppressive drugs and pp65 stimulation *in vitro*.** Representative dot plots show all CD8 T cells plotted for expression of CD107a against TNF. Top panel shows positive (pp65 only) and negative (DMSO only) controls. CD8 T cells after pre-incubation with three different concentrations of either CSA (40ng/ml, 200ng/ml and 1000ng/ml) (center panel) or FK506 (2 ng/ml, 10 ng/ml and 50ng/ml) (lower panel) and stimulation with pp65 are shown.

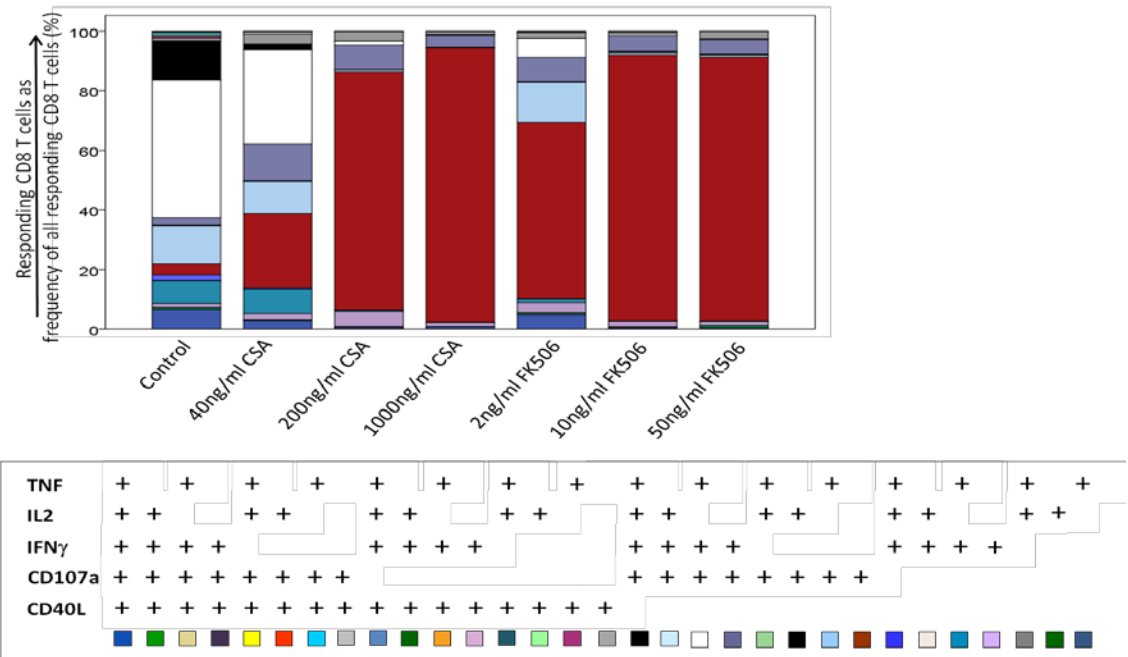


Figure 54: **Detailed cytokine profile of pp65-specific CD8 T cells after incubation with immunosuppressive drugs and pp65 stimulation *in vitro*.** Stacked bars show the frequencies of all responding CD8 T cells producing different combinations of activation markers after pre-incubation with three different concentrations of either CSA (40ng/ml, 200ng/ml and 1000ng/ml) or FK506 (2 ng/ml, 10 ng/ml and 50ng/ml) followed by stimulation with pp65. All samples were done in duplicates (n=3).

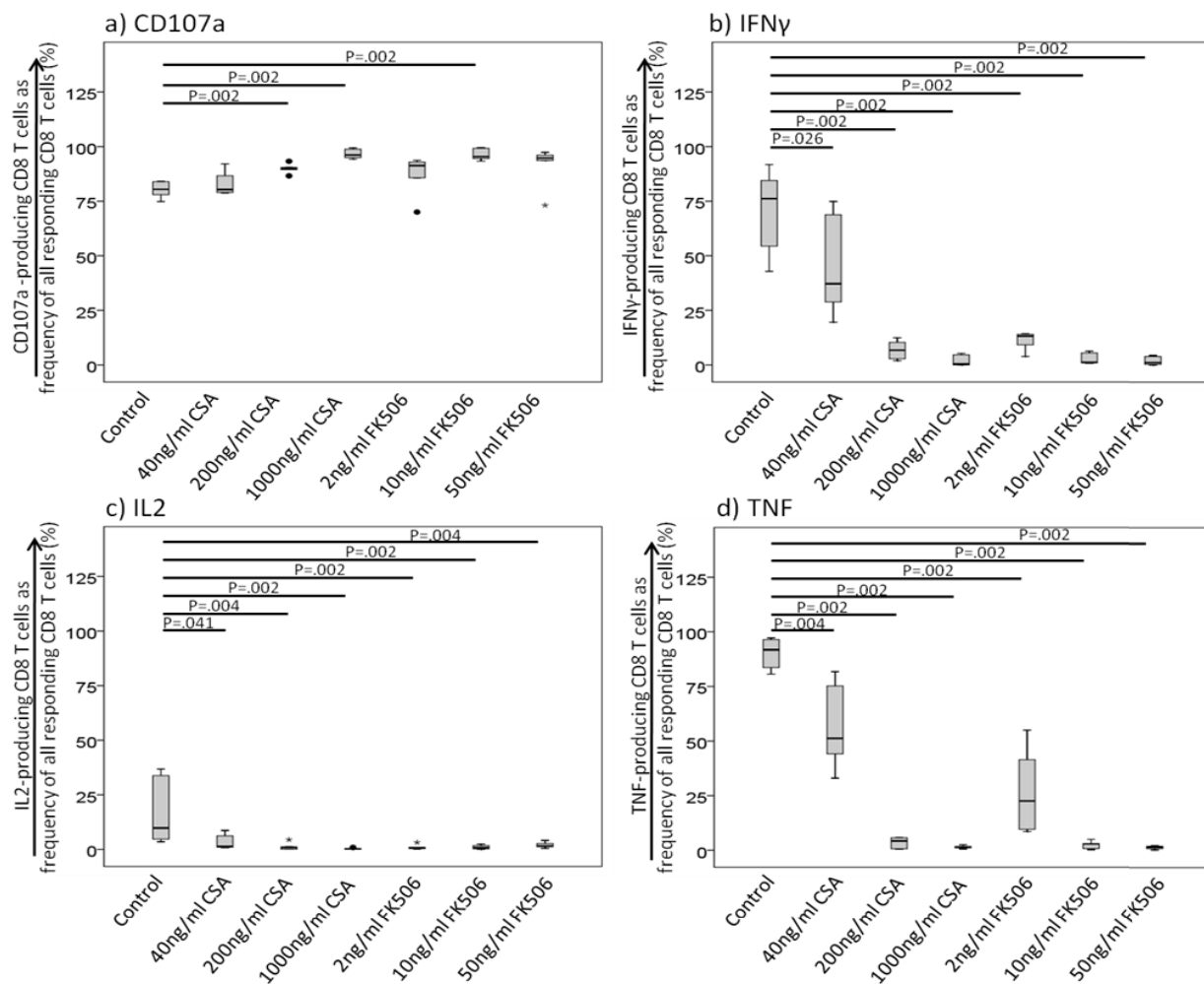


Figure 55: **Frequencies of cytokine expressing CD8 T cells after incubation with immunosuppressive drugs and pp65 stimulation *in vitro*.** Box plots show the frequency of CD8 T cells producing a) CD107a, b) IFN γ , c) IL2 and d) TNF in percentage of all responding CD8 T cells after stimulation with pp65 and pre-incubation with three different concentrations of either CSA or FK506. All samples were done in duplicates (n=3).

The frequencies of cells producing IFN γ , IL2 and TNF were reduced after pre-incubation with CSA or FK506. Furthermore, the amount of IFN γ and TNF produced per cell was decreased (Figure 56). The MFI of CD40L was not analysed because only very few CD8 T cells expressed it. The amount of IFN γ or TNF per cytokine-producing T cell was significantly reduced in cells pre-treated with all three concentrations of FK506 and the two highest concentrations of CSA. There were no differences regarding the MFI of CD107a or IL2 in cells pre-treated with or without immunosuppressive reagents.

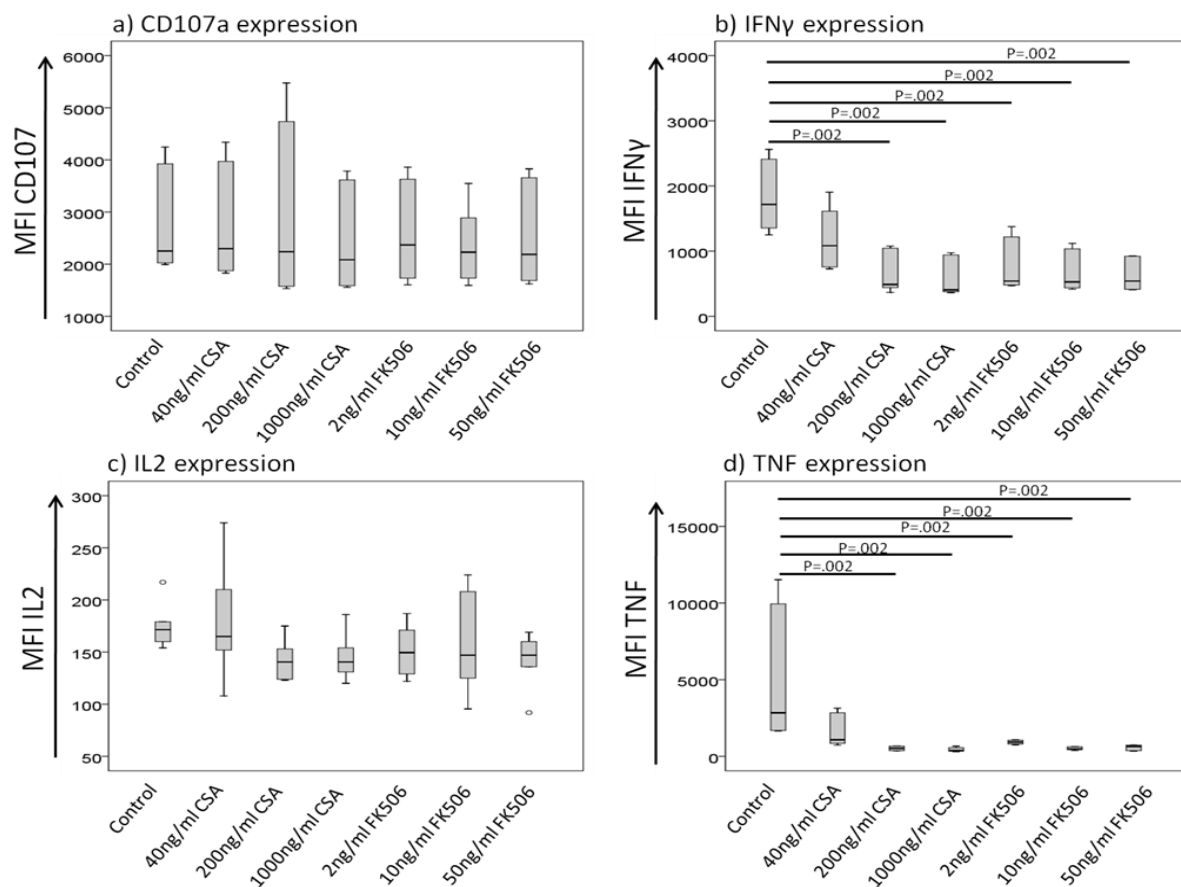


Figure 56: **MFIs of cytokine expressing CD8 T cells after incubation with immunosuppressive drugs and pp65 stimulation *in vitro*.** Box plots show the MFIs of CD8 T cells producing a) CD107a, b) IFN γ , c) IL2 and d) TNF after stimulation with pp65 and pre-incubation with three different concentrations of either CSA or FK506. All samples were done in duplicates (n=3).

In brief, the immunosuppressive drugs CSA and FK506 suppressed the production of IFN γ , IL2 and TNF in pp65-specific CD8 T cells but degranulation remained unaffected. Both the number of cytokine-producing T cells and the quantity of cytokine secreted per cell were reduced after pre-incubation with CSA or FK506.

6.7 Summary of results

This study provided the following results:

With advancing age of the donor, the memory T cell subsets changed towards further differentiated T cell subsets and these showed a downregulation of TCR and co-receptors compared to the least differentiated T_N cells.

CMV-infection altered the memory subset distribution in the entire T cell compartment and these changes were correlated with the size of the pp65-specific T cell response. Large pp65-specific responses were associated with marked losses and gains in T_N and T_{EM} cell compartments, respectively, small responses were associated with negligible changes.

The frequency of responding T cells to stimulation with CMV peptide pools pp65 and IE1 or the polyclonal stimulus, OKT3, increased with age of the donor. Polyfunctionality was increased in large CMV-specific T cell responses.

Polyfunctional T cells expressed quantitatively and qualitatively more activation marker on a per cell level than monofunctional cells and showed increased TCR and co-receptor downregulation upon activation.

In addition, CMV infection appeared to affect unrelated immune responses. CMV-infected donors displayed higher numbers of activated T cells of a more advanced differentiation phenotype following polyclonal or PPD-specific stimulation.

Polyfunctional pp65-specific T cell responses were inhibited by *in vitro* incubation with immunosuppressive drugs whereas degranulation of T cells was not affected.

7 Discussion

7.1 T cells

To gain more insight on the complex interaction of CMV infection and the human immune system, CMV-induced alterations in the T cell compartment of individuals in different age groups were analysed. PBMC from 50 donors were stimulated *in vitro* with a range of antigens and mitogen (pp65, IE1, PPD and OKT3) and the phenotype markers CD45RA and CD27 as well as the effector functions CD40L, IFN γ , IL2 and TNF were examined by multicolour flow cytometry.

CD4 and CD8 T cells (defined by the lineage markers CD3, CD4 and CD8) were analysed to investigate whether changes occurred as a consequence of age. Absolute counts and frequencies of CD3, CD4 and CD8 T cells and CD4/CD8 T cell ratios did not differ significantly between the three age groups. This is in accordance with other published results that showed no significant age-related differences of lymphocyte populations in healthy adults [107, 130-132]. However, most published data are inconsistent, showing either a decrease or no differences for absolute numbers and frequencies of CD3 T cells and absolute numbers of CD4 T cells with age [94, 117, 133-143]. The data also show an increase or no difference for frequencies of CD4 T cells and a decrease, an increase or no changes for the absolute counts and frequencies of CD8 T cells with age [94, 117, 133-143]. Most of these studies included more than 100 adult donors ranging from 18 -70 years. Only two studies included children, which alters the results since it is known that children have in, absolute terms, more CD4 and CD8 T cells, but less CD4 T and CD8 T cells in percent of all T cells than adults [94, 138]. Another reason for the discrepancies between the different studies might be the method. Some studies looked for correlations, others compared age groups with arbitrary cut-offs. Other methodological features, ethnic background, health status, environmental and genetic factors could also affect the results and account for the differences since the studies were conducted in different populations.

As described previously, absolute counts for CD3, CD4 and CD8 T cells were higher in CMV-seropositive than CMV-seronegative donors but only the differences for absolute CD3 and CD8 T cell counts reached statistical significance in this study [106-108, 144, 145]. This

resulted in a decreased CD4/CD8 ratio in CMV-infected donors as already published [106, 107, 109]. Of note, only one (young) donor in this study had a CD4/8 ratio smaller than 1.

In agreement with published reports, the frequencies of T cells and the absolute numbers per ml blood were highly correlated for all tested populations [146]. It is unclear, which parameter is more useful. For example, one donor may have very low T cell counts while another has very high T cell counts. The proportions of CD4 and CD8 T cells within this population may be identical. Hopefully, future studies will reveal which parameter is more closely related to disease outcome.

7.2 Memory T cell subsets

As they encounter their cognate antigen for the first time, CD4 and CD8 T_N cells are activated and start undergoing antigen-dependent differentiation. Analysis of the expression of T cell surface markers such as CD45RA, CD45RO, CD27, CD28 and CCR7 has led to several models describing this differentiation pathway [147, 148]. For example, Hamann *et al.* (1997) based their model on the expression of CD45RA and CD27 to distinguish between T_N, T_{CM}, T_{EM} and T_{EMRA} cells [14].

In this study the same markers were used to distinguish between the four memory T cell subsets. T_N cells express CD45RA, a surface glycoprotein which is involved in T cell signalling and CD27, a member of the TNF receptor family [149]. CD45 isoform expression (CD45RA or RO) is altered on T cells during and subsequent to activation. Whereas CD45RA is found on T_N cells, CD45RO is found on memory T cells [150]. Eventually, the most advanced memory T cells re-express CD45RA, which is why they are referred to as 'revertant' [151]. Although CD27 is not classified as a costimulatory molecule, it enhances responses by increasing T cell proliferation [16, 152, 153]. CD27 expression increases transiently with activation and is subsequently irreversibly downregulated on memory T cells after several rounds of cell division [154].

CD28, a costimulatory molecule, is another commonly used differentiation marker, [106, 112, 155, 156]. Appay *et al.* (2002) provided a differentiation model based on the expression of CD27 and CD28, which distinguished between early, intermediate and late differentiated T cells [157]. During normal antigenic exposure, CD28 expression is reduced but is rapidly restored to the same level as before stimulation. The loss of CD28 on T cells is attributed to

repeated antigenic stimulation. CD8 T cells lose CD28 first and then CD27 whereas CD4 T cells lose CD27 first and then CD28 [59, 158]. Virtually no CD8 T cells express CD28 without CD27 while CD4 T cells do not express CD27 without CD28. Therefore, all CD27⁻ CD8 T cells in this study can be assumed to be CD28⁻ T cells. CD28⁻ CD8 T cells in humans contain high frequencies of virus-specific functional memory T cells [159]. These T cells show enhanced cytotoxicity but impaired antigen-induced proliferation. In humans, all CD8 T cells express CD28 at birth but these cells decrease by half at the age of 80 years [160]. The resulting increase of peripheral CD28⁻ CD8 T cells with age is observed in primates and humans but not in mice [161]. The differences between human and murine immune systems and varying life spans are just two reasons why mouse models are of limited use in the study of immunosenescence. Furthermore, inbred mouse strains with single infections are not comparable to genetically diverse humans which undergo multiple infections during their lifetime. For these reasons, data generated on mice were only referred to if no data on humans were available.

Unfortunately, it is difficult to compare data regarding memory T cell subsets from different published studies since different markers which identify only partly overlapping subpopulations were frequently used [94, 114, 117, 136, 138, 149, 162, 163]. The phenotypic markers CD45RA and CD27 were used in this study to distinguish between T_N (CD45RA⁺/CD27⁺) and the three memory subsets: T_{CM} (CD45RA⁻/CD27⁺), T_{EM} (CD45RA⁻/CD27⁻), and T_{EMRA} (CD45RA⁺/CD27⁻) cells [14]. It was shown that the simultaneous analysis of the four memory markers CD45RA, CD27, CD28 and CCR7 characterised more than 10 different T cell populations and each additional phenotypic marker further increased the number of distinct T cell populations [162, 163]. For example, using multicolour flow cytometry to analyse 11 phenotypic markers resulted in 75 detectable T cell populations [149]. This multiplicity of memory T cell subsets suggests that the plasticity of these populations is greater than previously thought and that the analysis of just two markers significantly simplifies the picture. Given that the number of fluorescence channels, even on the most advanced machines, is limited, a compromise in the choice of markers has to be made.

Independent of which differentiation markers are used, it is generally accepted that T_N cells decline and memory T cells increase with age, as confirmed by the results shown in this study [94, 95, 114, 117, 136, 138, 164-166]. CD4 and CD8 T cell subsets underwent the same phenotypic shifts during ageing, but the changes were more pronounced in CD8 T cells. The

accumulation of memory T cells in older people can be explained by the reduced thymic output of T_N cells, frequent activation of T cells and the fact that, T_{EM} and T_{EMRA} cells are more resistant to apoptosis than T_{CM} and T_N cells [167-171]. In addition, T_{CM} and T_N cells from old people were more sensitive to apoptosis than those from younger people, resulting in a further decrease of these T cell subsets in elderly people [168].

The four memory T cell subsets were further investigated with regards to TCR downregulation upon activation. The activation induced loss of CD3, CD4, and CD8 co-receptor expression was used as a surrogate marker for TCR downregulation. The expression difference was determined by comparing the MFIs of CD3, CD4 and CD8 on the different memory subsets of unstimulated T cells. It was observed that CD3, CD4 and CD8 downregulation was more pronounced in more advanced memory subsets compared to T_N cells. The same level of downregulation was seen across different age groups. It is known that successful activation of the T cell results in internalisation and degradation of TCR, CD3 and co-receptors [23, 172]. This TCR downregulation after activation is usually reversible to enable recurrent stimulation of T cells. The downregulation of TCR and co-receptors on T cells of advanced differentiation seen in this study indicates that subsequent T cell stimulation results in irreversible downregulation of TCR and co-receptors.

CMV infection is known to alter the memory subset distribution of all T cells, irrespective of their specificity [107, 111-113, 119]. Memory T cells, which play an important role in viral control, are stimulated and proliferate each time CMV is reactivated. However, CMV is never eradicated by this response [92, 93]. In this study, the T_{EM} and T_{EMRA} cell subsets were proportionally bigger and the T_N subset was proportionally smaller in CMV-infected people than in uninfected people. This was observed for CMV-specific T cells as well as the entire T cell population. These alterations were more pronounced in CD8 T cells than in CD4 T cells and more conspicuous in elderly people than in young people.

The results of this study extend these observations by showing that this effect depends on the size of the pp65-specific T cell responses and not on the presence or absence of CMV infection per se. The bigger the pp65-specific T cell response was, the less T_N and the more T_{EM} cells were observed in the peripheral blood. It appeared that the pp65-specific response size was a better correlate for the overall memory T cell subset shift than the age of the donor, which is used in most studies. This is an important finding, suggesting that the way in which the immune system deals with pp65 determines whether the effect on the T cell compartment is

weak (small pp65-specific T cell response) or strong (large pp65-specific T cell response). Of note, the size of the IE1-specific CD8 T cell response was not correlated with an advanced memory distribution of the entire CD8 T cell compartment. It appears that the more abundant, structural pp65 protein has a stronger effect on shaping the immune system than the non-structural IE-1 protein. It is still unclear, why large pp65 or IE1-specific T cell responses develop in some, but not other, individuals. Unfortunately it remains unclear whether large T cell responses to CMV invariably occur after many years of infection, or if some individuals simply never mount these large responses.

In this study, the frequency of CD8 T_{EMRA} cells was significantly correlated with age in both CMV-negative and CMV-positive individuals. Although T_{EMRA} cells were thought to be associated with CMV infection, they have been found in other settings as well e.g. after yellow fever vaccine and in patients with tuberculosis [18, 173-175]. CMV-specific CD8 T cells are CD45RO during primary infection and some convert to CD45RA T cells later on [151]. CD45RA re-expression has been positively correlated with the time since clearance of the virus from blood after primary CMV infection in healthy people and with the duration of CMV replication in transplant patients [176, 177]. The frequency of T_{EMRA} cells increased after primary infection in the first year and then reached an amount comparable to those found in long-term, latently infected individuals. It also has been demonstrated that high frequency of T_{EMRA} cells seemed to be associated with control of HIV replication and a lower risk of intrauterine CMV transmission [176, 178]. T_{EMRA} cells were initially described as highly cytotoxic terminally differentiated T cells with short telomers and therefore poor proliferative capacity [179-181]. Other publications showed that the telomeres were longer than in other memory subsets and that T_{EMRA} cells proliferated upon antigenic stimulation [182-184]. The differentiation pathways and functions of this memory T cell subset have to be investigated further.

Although there is no direct evidence that a highly differentiated T cell compartment has a negative impact on the immune response, individuals with a low frequency of T_N cells are thought to be more susceptible to new infectious agents. The accumulation of highly differentiated CD8 T cells is one of the parameters included in the IRP, which predicted mortality in the elderly in a number of longitudinal studies [98, 99]. The IRP was defined by the Swedish longitudinal OCTO/NONA studies and also included poor proliferative T cell responses to mitogens, a CD4/CD8 T cell ratio of less than one and CMV-seropositivity.

Another study established that an inverse CD4/CD8 T cell ratio (i.e. <1) is associated with poor survival in elderly people [185]. Due to a significant positive correlation between the proportion of CD8 T cells and age, the CD4/CD8 ratio decreases in older people [139, 186]. Interestingly, it now appears that most immune parameters defining the IRP are influenced by CMV infection, but not by other persistent viral infections, e.g. EBV [187]. The mechanisms by which CMV infection might reduce survival in some elderly people are still unclear. Although 100% of people with an IRP were CMV-seropositive, 85% of the people without an IRP were CMV-positive as well [188]. This clearly points to an important role of host factors in determining the effect of CMV on the immune system. It would be interesting to analyse if the pp65-specific response size and therefore the CMV-driven shift in memory subsets is the same in elderly with or without an IRP. This may explain why only some people with CMV infection are in the IRP group. It seems that the manner in which the immune system of an individual handles the virus might be crucial rather than simply infection status. This explanation is supported by the results of this work, indicating that the way the immune system deals with the infection is more important than CMV infection itself. In agreement with this theory a recent publication suggests that individuals genetically enriched for longevity are less susceptible to CMV-associated age-driven immune alterations, which might reflect better immunological control of the virus and contribute to their decreased mortality rate [189].

7.3 Polyfunctional T cell responses

Studies of viral infections have highlighted the phenotypic and functional heterogeneity of antigen-specific T cells and recent studies suggested that T cells which express a multitude of effector functions simultaneously are more likely to be correlated with protective immunity than single cytokine producers [124-128, 190, 191]. In this study, a multicolour flow cytometric approach was used to identify phenotype and functional profiles of CMV-specific T cells. The expression of the activation markers IFN γ , IL2, TNF and CD40L in addition to the phenotype markers CD45RA and CD27 on CD4 and CD8 T cells in terms of proportions of T cells and absolute cell counts per ml blood were analysed.

Several tools are currently available for the detection of specific T cells by flow cytometry (rev. in [29, 192, 193]). Methods include intracellular cytokine staining (ICS), MHC class I tetramers and dextramers, measurement of proliferation by CFSE or PKH dyes, phosphorylation and degranulation assays [33-39]. Several of these methods can be combined

to gain insight into antigen-specific immune responses and the methods have sufficient sensitivity to measure rare cell populations. This is important since antigen-specific T cell responses are usually very small. The measurement of intracellular cytokines in PBMC after stimulation was first done by Waldrop *et al.* (1997) to analyse CD4 T cell responses to CMV-lysate [40]. The most commonly used parameter to investigate functional CMV-specific T cell responses is the frequency of either CD4 or CD8 T cells producing IFN γ after short-term stimulation [118, 146, 194-204]. Although IFN γ plays an important role in the control of infections, the frequency of IFN γ -producing T cells does not necessarily appear to be a good correlate of protection on its own [124, 205]. Recent studies suggest a critical role of qualitative differences in the T cell response for the prediction of disease outcome, assigning a protective role to subsets that are able to produce several cytokines simultaneously [124-129, 190, 191, 206]. Therefore, four activation markers (IFN γ , TNF, IL2 and CD40L) were analysed in this study. TNF is an important cytokine for fighting infections by mediating killing of intracellular pathogens [1]. IL2 promotes proliferation of effector T cells, whereas the expression of CD40L identifies mainly activated CD4 T cells that can activate dendritic cells [1, 30, 31]. T cells producing CD40L, IFN γ , IL2 or TNF in any combination after antigen stimulation are referred to as antigen-specific T cells. T cells expressing only one of the four activation markers were considered monofunctional T cells and T cells expressing more than one of the analysed activation markers were considered polyfunctional for the purpose of this study.

Unfortunately, even with high-end flow cytometers, technical limitations are quickly reached when it comes to the analysis of complex T cell responses. For example, this study used an instrument able to detect 11 fluorescence colours, but difficult choices still had to be made. An 11-colour staining panel including one 'dump channel' (to exclude specific unwanted cell populations), a viability dye, three T cell lineage markers (CD3, CD4 and CD8) and two phenotypic markers (CD45RA and CD27) allowed analysis of four activation markers. A dump channel, viability dye and lineage markers were considered essential, and at least two memory markers are required for achieving a reasonable distribution of T cell memory subsets. The use of four carefully chosen activation markers is likely to have identified the majority of polyfunctional T cells but may have missed some polyfunctional subsets altogether or 'misidentified' them as monofunctional. This will have included cells upregulating markers other than those measured here. New T cell activation markers are continually identified that may have important roles in different infections. So far, mainly IFN γ has been used to identify

functional CMV-specific T cells but recent publications propose that chemokines like MIP-1 α and MIP-1 β might be expressed on more T cells and might therefore be better markers, especially for terminally differentiated T cells [207-209]. Another important feature in the antiviral immune response is degranulation of T cells which can be detected by CD107, however, for the technical limitations described above it was not included in the panel [210].

Conclusions of this study are in agreement with other publications showing that the CD8 T cell response to viral proteins is larger than the CD4 T cell response (rev. in [120]). This may be because proliferative capacity is higher in CD8 T cells or because antigen presentation is more efficient for CD8 T cells than for CD4 T cells (rev. in [120]). In this study there was no significant correlation between the pp65-specific CD4 and CD8 T cell response, in contrast to other studies where a positive or negative correlation was found [211, 212].

Some of the discrepancies between reports might be related to the small numbers of donors per study and to methodical differences, such as addition of growth factors co-stimulation and the source of T cells e.g. fresh or frozen cells, PBMC or sorted cells. Although a variety of techniques like intracellular flow cytometry, MHC tetramers and ELISPOT enable the investigation of CMV-specific immunity, it is still not possible to enumerate all CMV-specific T cells. Stimuli such as peptide pools or tetramers identify just fractions of all CMV-specific T cells. Even the use of whole CMV-lysate will not identify all CMV-specific T cells, since CD8 T cells are not efficiently stimulated [213]. Stimulation with CMV-lysate and peptide pools also requires a functional read-out and this limits the detection to only functional T cells. The use of tetramers also identifies dysfunctional cells but is epitope and HLA-restricted and might not work for all antigens [214]. Another important consideration is, that these studies only analyse T cells in the peripheral blood and T cells responsible for controlling or eradicating viruses might be in other tissues, e.g. bone marrow or lymph nodes [215-218]. During this study it became evident that the use of different fluorochrome-Ab conjugates also affect results in the same donor. The use of the same reagents and regular performance checks of the cytometer throughout the whole study allowed for direct comparisons between donors within this study but comparisons with the results of other studies may be limited for the above mentioned reasons.

The results of this study showed an increase of the frequencies and absolute numbers of pp65-specific CD4 and CD8 T cell response size with ageing, but this was not statistically significant for all correlations. As previously demonstrated frequencies of both CMV-specific

CD4 and CD8 T cells showed a wide range among CMV-seropositive individuals in this study [52, 118, 201, 219, 220]. Large responses also occur in young and middle aged people and it is likely that the duration of CMV infection in combination with other host factors (rather than simply age) determines response size in an individual. No correlations with age were found for IE1-specific CD4 or CD8 T cell responses. As previously observed, IE1-specific CD4 T cell responses in this study were very small in most donors and therefore excluded from further analysis [52, 56, 204]. The increase in response size might not correlate in a linear fashion with time since infection, but is likely to be a better correlate of it than age which is used in most studies. Only one study in a Sicilian population showed no increase in CMV-specific tetramer-positive T cells with age [221]. In contrast, most cross-sectional studies showed that the CD4 and CD8 T cells specific for pp65, IE1 and CMV-lysate increased in CMV-seropositive donors with advancing age [118, 119, 144, 212, 222-225]. Although it was shown that old people have increased frequencies of CMV-specific T cells it is still unclear, when this T cell expansion happens. For obvious reasons, so far no longitudinal study in healthy humans has been conducted to follow the time course of infection for more than one year. The investigation of primary CMV infection in healthy adults is very difficult because the time point of infection is usually unknown. Although CMV infection is mainly acquired during infancy and adolescence, the rate of CMV seroconversion in adults is still ~1% per year [48]. Therefore it is generally not possible to determine the length of time that individuals have been infected for. Studies in transplant patients and pregnant women with primary CMV infection showed that after an initial peak of CMV-specific T cells during primary CMV infection, the frequencies of CMV-specific T cells remained stable, at least for one year [108, 176, 198, 201, 220, 226, 227]. One study monitored the responses to staphylococcal enterotoxin B (SEB) and CMV antigens for 6 month in healthy CMV-seropositive donors [93]. While the response to SEB was relative stable over time, the T cell responses to CMV-lysate and pp65 showed significant variability [93]. Although the response size varied, there was no trend towards an increase with time. This seems to be in conflict with studies showing an accumulation of CMV-specific T cells with advancing age. One explanation could be that the expansion happens constantly but on such a low level, that no differences can be detected in the time course of one year (if it were 5% of the response size per year, it would not be measurable after one year, i.e. 1% versus 1.05%, but after 10 years it would be 1.6%, i.e. a 60% increase). Another possibility might be that the expansion happens infrequently, especially at older age after reactivation of CMV [228]. It is also possible that the CMV-specific T cell response size increases

infrequently in individuals after exposure to exogenous CMV. For example, HIV-specific T cell responses in HIV-infected individuals increased with frequent exposure to exogenous HIV [229]. It has to be investigated, why some people display large CMV-specific T cell responses while others only maintain small T cell responses which seem to be sufficient to control CMV.

Although it is generally accepted that CMV-specific T cells increase with age, it is still uncertain if the majority of these T cells are functional or not in older people. Few studies reported an accumulation of functional CMV-specific T cells in the elderly, but several studies suggested a loss of functional CMV-specific T cells in elderly people [99, 109, 118, 119, 144, 212, 222-225]. These T cells, in particular CD8 T cells, have sometimes been described as dysfunctional T cells that supersede functionally more diverse T cells so that the immune system becomes less able to respond to challenges requiring T cell responses. Because functional readouts were used to detect and enumerate CMV-specific T cells in this study, it was not possible to analyse non-functional CMV-specific T cells. The results showed that the absolute numbers and frequencies of functional T cells after *in vitro* stimulation with pp65 and IE1 increased with advancing age. In addition, polyfunctional CD4 and CD8 T cells were increased in large pp65-specific responses. Irrespectively of response size or age a robust population of polyfunctional CMV-specific T cells was present and may be in control of CMV. The inference that in individuals with large CMV responses, T cells are dysfunctional, and, as a result, more of them are required to control CMV is not supported.

All four analysed activation markers, IFN γ , IL2, CD40L or TNF in the T cell compartment were increased after pp65 stimulation with advanced age and every single marker was significantly correlated with the response size. In contrast to other studies, this study also observed an increase in IL2 producing T cells with age [208, 225, 230, 231]. Nevertheless, IL2-producing T cells accumulated less than cells expressing any of the other markers. Highly differentiated T cells lose the ability to produce IL2 as a consequence of the loss of the costimulatory receptor CD28 [14, 232]. The majority of CMV-specific T cells are highly differentiated and do not express CD28 [226, 233, 234]. This observation explains why fewer CMV-specific T cells express IL2 than other cytokines examined. In conclusion, this study does not confirm a loss of fully functional T cells with increasing response size or age if either all CD4 T cells or all CD8 T cells are used as the reference subset. The same observation was also seen if absolute counts in cells per ml blood were used. It cannot be ruled out that non-functional T cells (not detected in this study for reasons discussed above) increase at a higher

rate than the polyfunctional T cells, but it can be excluded that non-functional T cells replace polyfunctional T cells.

Since multicolour flow cytometry is a relative new technique and there is a multitude of possible markers, the analysis of data has not been standardised (and maybe never will be). Some efforts are being made to streamline reporting of experiments to make differences transparent [235]. It is important to note that the reference population in terms of defining an increase or decrease makes a significant difference. The use of the CD4 or CD8 population, or even absolute counts per ml blood as a reference measure the increase or decrease of certain specific subsets of a response, irrespective of other independent subsets which are part of that response. Several studies report a relative loss of functional CMV-specific T cells in elderly CMV-positive donor [109, 118, 222, 223]. At the same time, these studies report higher numbers of CMV-specific T cells with advanced age. Taken together, most of these published data could actually be interpreted to show a total increase of functional CMV-specific T cells with age which is in agreement with the results presented here [109, 118, 222, 223].

Polyfunctionality was also analysed with respect to the four memory T cell subsets defined by CD45RA and CD27 expression. With pp65 and IE1 being immunodominant CMV-proteins, it was also of interest to analyse the T cell response to other stimuli, such as PPD and OKT3. Regardless of the stimulus, only small frequencies of T_N cells were activated. The use of the two markers CD27 and CD45RA in this study did not define a pure T_N cell population. The contamination with activated and/or differentiated T cells may have been as high as 13% [149]. For example, these T cells might be activated CD27⁺/CD28⁻/CD45RA⁺ T cells [163, 184]. This explains why some of the T cells that were classified as T_N cells in this study produced activation markers upon stimulation. A minimum of three differentiation markers is required to identify true T_N cells which would not be expected to produce cytokines after short-term stimulation with more than 95% accuracy [149]. Most activated T cells were observed in the T_{CM} and T_{EM} cell subsets for all used stimuli. CMV-specific CD4 and CD8 T cells and polyclonally stimulated CD4 T cells were mainly T_{EM} cells. In comparison, PPD-specific CD4 T cells and polyclonally stimulated CD8 T cells were primarily observed in the T_{CM} cell subset. Supposing that polyclonal stimulation activates T cells of all specificities, the phenotype of these cells would present an average. CMV-specific T cells would be of a more advanced memory phenotype than the average, while PPD-specific T cells would be of a less advanced memory phenotype than the average. This is in accordance with other studies

showing that T cells specific for different pathogens display distinct phenotypes and effector functions [110, 157, 158, 162, 236-238].

CD8 T_{EMRA} cells were found to be activated after stimulation with pp65 and IE1. Previous research has found that CMV-specific CD8 T cells accumulate in the T_{EMRA} cell subsets with average percentages as high as 50% of all CMV-specific T cells [176, 177, 215]. In this study, up to 70% CD8 T_{EMRA} cells were seen, but the average for CD8 T_{EMRA} cells was only ~14% of all pp65 and IE1-specific CD8 T cells. As observed for all responding T cells, there were large inter-individual variations regarding the memory T cell subset distribution of activated T cells.

While T_N cells were, for the most part, monofunctional regardless of the stimulus used, the polyfunctionality of the other three memory subsets was dependent on the stimulus. In general, antigen-specific stimulation with CMV-antigens and PPD resulted in a more polyfunctional T cell response than polyclonal stimulation with OKT3. Stimulation with pp65 induced high frequencies of polyfunctional T cells in all three memory T cell subsets (T_{CM}, T_{EM} and T_{EMRA}), while less IE1 stimulated T cells were polyfunctional. PPD induced only polyfunctional T cells in the T_{CM} and T_{EM} subsets. More CD4 T cells than CD8 T cells produced three or four activation markers simultaneously because CD40L is mainly expressed on activated CD4 T cells. As mentioned above, the measurement of four activation markers cannot provide a complete picture of polyfunctional T cells. Nonetheless, these data suggest that the polyfunctionality of T cells is not only dependent on the differentiation of T cells but also on the stimulus. This is in agreement with the findings that polyfunctionality of T cells depends on different factors such as the type of antigen, APC, antigen concentration and antigen-dependent differentiation stage of the T cell [125, 210, 230, 239-241]. In T cells with higher antigen-sensitivity antigen contact results in a stronger signal than in T cells with lower antigen-sensitivity, and a stronger signal then translates into a more polyfunctional profile [125].

TCR downregulation directly follows T cell activation and the degree of TCR downregulation is known to correlate with antigen-sensitivity [42, 172, 241-243]. As a result, one would expect TCR downregulation to be directly linked to polyfunctionality and the results shown here confirm exactly this. Activation induced CD3 downregulation (as a surrogate for TCR downregulation) directly correlated with the number of upregulated activation markers. The CD3 and CD4 surface expression on CD4 T cells and the CD3 and CD8 surface expression on CD8 T cells were downregulated on the surface of activated CMV-specific T cells. The

downregulation of these surface molecules was more pronounced when the number of different cytokines produced per cell was higher.

The results presented here show that T cells producing multiple cytokines simultaneously also express more cytokine per cell than the equivalent single producers, supporting the idea that they might be more efficient [21, 120, 124, 240]. This was observed for CD4 and CD8 T cells after stimulation with PPD, CMV peptides and OKT3, indicating that it is a general feature of T cells independent of the stimulation. The more activation markers a cell expressed, the higher the quantity of CD40L, IFN γ and TNF per single cell. The situation was different when the expression of IL2 was analysed. Although subsets expressing IL2 in combination with one other cytokine were very small, these T cells produced significantly more IL2 per cell than monofunctional T cells or T cells producing three or four activation markers. Moreover, the expression levels of IL2 were consistent, irrespective of the activation marker that was being co-expressed. It is unclear, why the pattern of IL2 expression in polyfunctional T cells is different from the expression patterns of TNF, IFN γ and CD40L. It appears that IL2 plays an important role in the early stages after antigen encounter, in particular with respect to proliferation, indicating that T cells at that stage do not need to be polyfunctional. Further studies investigating more activation markers may help to better understand expression patterns of polyfunctional T cells.

In conclusion, this study does not confirm a loss of T cell functionality with increasing response size and/or age. The pp65-specific T cell responses clearly suggest that bigger responses include more polyfunctional T cells. The results indicate that response size rather than donor age might be a better indicator of the duration of CMV infection. It certainly appears to be more closely correlated to other observable changes within the T cell response (subset distribution and polyfunctionality) rather than donor age. Since the CMV-specific T cell response size increases over time after CMV infection, it is likely there will be a correlation with age. This study supports the notion that host factors regulating CMV-specific T cell response size have a crucial role in determining the extent to which CMV affects the cellular immune system in general.

7.4 Effect of CMV on unrelated T cell responses

Infection with CMV emphasises the effects of ageing on the immune system and is known to impair the immune system in various ways. Nevertheless this work showed that CMV infected individuals had bigger T cell responses to antigen-specific (PPD) and polyclonal (OKT3) stimuli than uninfected individuals. PBMC from CMV-infected and uninfected donors were stimulated overnight with protein purified derivative (PPD) from *Mycobacterium tuberculosis* or OKT3 and subsequently analysed by flow cytometry. Expression of the phenotype markers CD45RA and CD27 and the effector molecules CD40L, IFN γ , IL2 and TNF were examined.

PPD was used as a recall antigen to analyse CMV-unrelated antigen-specific T cell responses. Over 2 billion people are infected with *M. tuberculosis* and more than 85% of all children worldwide are vaccinated with Bacillus Calmette-Guérin (BCG), a vaccine against tuberculosis that is prepared from a strain of attenuated live *Mycobacterium bovis* [244, 245]. Therefore, most people show a specific CD4 T cell response to PPD and these T cells express IFN γ , IL2 and TNF [246, 247].

CMV infected donors had higher absolute numbers of PPD-specific CD4 T cells per ml blood compared to uninfected donors but the frequency in terms of CD4 T cells was similar in both groups. No CD8 T cell response was detected after stimulation with PPD. The responding CD4 T cells showed a similar cytokine pattern in CMV-infected and uninfected individuals whereas the memory T cell distribution changed. CMV-seropositive people had both proportionally and in absolute numbers more responding PPD-specific T_{EM} cells after stimulation. This is consistent with previous studies showing that T cells specific for bacterial (PPD) and other viral (HSV, EBV) antigens are more differentiated in CMV-positive donors than in CMV-negative donors [248]. If these cells are of advantage or disadvantage for the host has to be investigated.

It is well known that CMV infection impairs the immune system, but only a few studies have been carried out that focus on the influence of CMV on co-resident infections in humans. In mice it is described that murine CMV protects mice from bacterial infection [249]. The authors suggest that enhanced levels of T cell activation and secretion of IFN γ due to the chronic presentation of viral antigens protects the host from subsequent bacterial infections. In humans during acute CMV infection, the absolute numbers of EBV specific CD8 T cells dropped temporarily and recovered [108]. This is in contrast to a study conducted by Khan *et al.* (2004)

that demonstrated that CMV infection impaired the response to a coresident EBV infection permanently [118]. CMV infection also impairs the response to influenza vaccine in elderly people [250]. It was recently published that the total number of CD8 T cells increases with immunological experience, e.g. vaccination but impairs pre-existing T_{EM} cells [251, 252]. In this study, the PPD-specific cells are not impaired by CMV infection but in contrast the PPD-specific T cell response is actually enhanced. About 60% of CMV-infected CD4 T cells in HIV-infected donors produce MIP-1 α and MIP-1 β and are therefore protected against HIV infection [118, 207]. Nonetheless, CMV infection seems to enhance the progression of AIDS and CMV viral load has a strong association with mortality in patients with late-stage AIDS [70, 253]. In contrast, recent studies show that CMV and EBV-specific T cells contribute to antiviral T cell responses in humans [254, 255]. In these studies the T cell response to primary viral infections such as hepatitis B, HIV and dengue virus consisted of up to 20% of EBV and CMV-specific activated T cells. It is not yet known which mechanisms underlie this unspecific activation. One possible explanation may be the occurrence of TCR sharing between different T cells [256]. The contribution to immune responses to heterologous viral infections indicates an advantage of CMV infection for the human host [254, 255]. The CMV-specific T cells that were activated by other viral infections were found after primary infection *in vivo* and it is unclear if such activation was due to virus-specific factors or inflammation in general and if this could also happen after *in vitro* short-term stimulation. In this study, CMV-infected donors had larger responses to stimulation with PPD in terms of absolute numbers and it is possible that some of these activated T cells were CMV-specific. Another explanation is the induction of an inflammatory environment by CMV which drives also proliferation and differentiation of T cells specific for other pathogens [248]. This would also explain why CMV-seropositive donors, in contrast to CMV-negative donors, have higher spontaneous cytokine production without antigenic stimulation, which was demonstrated in this work and in other previous studies [36, 93].

CMV-infected people compared to uninfected people had also significantly more CD8 T cells both, in absolute numbers and proportionally, responding to polyclonal OKT3 stimulation. The number of responding CD4 T cells following OKT3 stimulation was not altered. Activated CD4 and CD8 T cell compartments contained significantly more T_{EM} and T_{EMRA} cells and appeared to be more polyfunctional in CMV-infected donors compared to uninfected donors. This is in line with published reports that demonstrated that more T cells from CMV-infected donors were activated in response to polyclonal stimuli (SEB, PHA) than T cells from

uninfected donors [106, 132, 145, 257, 258]. In other studies the frequencies of T cells responding to SEB and PMA/Ionomycin stimulation were similar in CMV-infected and uninfected people [95, 164, 222]. The influence of CMV infection on polyclonal T cell response remains controversial and needs further investigation.

Due to the fact that most studies have analysed only pp65 and IE1-specific T cells it remained unclear whether the higher number of memory T cells in CMV-infected people was only due to an increase of CMV-specific memory T cells or if CMV was changing the entire immune response. This study suggests that the immune response to unrelated antigens was enhanced indicating that CMV alters the whole immune system by inducing an inflammatory environment.

7.5 Effect of immunosuppressive drugs on CMV-specific T cell response

CMV is still the major infectious complication after solid organ transplantation, affecting more than 50% of all patients and causing both direct and indirect morbidity [73-75]. Direct effects caused by the virus include nephritis, hepatitis, colitis and myocarditis. Indirect effects are caused by the interaction of the virus and host immune system and are associated with graft rejection, opportunistic infections and decreased recipient survival [72, 76].

Solid organ transplant recipients have a high risk of CMV disease not only due to the immunosuppression but also because of immune system activation, triggered by the foreign organ. Immunosuppression itself does not reactivate the virus but prevents control of the activated virus by inhibiting the CMV-specific T cell response [259]. CMV-reactivation can be triggered e.g. by stress, immune activation and some cAMP-elevating drugs [259]. The level of immunosuppression required depends on the transplanted organ since different transplant organs induce different levels of immune activation. In contrast to heart transplant patients or heart-lung-transplant patients, liver and kidney-transplant patients receive a lower dose of immunosuppression and can better control primary CMV infection [211]. Most liver transplant patients are treated with either CSA or FK506 to prevent rejection whereas heart, heart-lung, or lung recipients receive triple therapy (Calcineurin inhibitors, steroids and antiproliferative drugs) [260, 261].

In order to avoid unnecessary antiviral treatment of transplant patients with a low risk of CMV-disease, it is important to find immunological (e.g. specific T cell subsets) correlates of protection against CMV-disease. For this purpose it is important to understand how immunosuppressive drugs are affecting CMV-specific T cells. Different markers of immunity against CMV were proposed to be correlated with the clinical outcome of CMV infection. So far, no test is routinely used which can predict CMV disease. Viral load measured by PCR is not always associated with CMV disease [59, 262]. Sund *et al.* (2009) showed that the reduction of IFN γ -producing CD4 T cells during the first few months after transplantation correlated with the magnitude of the CMV DNAemia [200]. They proposed to use the relative reduction of T cell responses after transplantation since the absolute number varies hugely between individuals [200]. Other groups stated that the ratio of pp65-specific IFN γ -secreting CD8 T cells to tetramer-positive CD8 T cells is correlated with protection against CMV disease or that the time point of the first CD4 T cell response, and not the quantity of the CD4 T cell response, is predictive for CMV disease [50, 199]. Additionally, plasma levels of cytokines and chemokines were investigated and high levels of IL8, as well as low levels of MIP-1 α , were found to be associated with CMV disease [263]. The total leucocyte count decreased prior to an increase of CMV viral load in heart transplant patients with CMV disease [264]. To date, none of the aforementioned immunological markers has been generally accepted to predict the risk of CMV disease.

To find out how immunosuppressive drugs affect CMV-specific T cell responses, PBMC from healthy CMV-seropositive donors were incubated *in vitro* with CSA and FK506 and subsequently stimulated with pp65. Calcineurin inhibitors were selected for these experiments since most transplant patients receive them. The activation markers CD40L, IFN γ , IL2, TNF and CD107a were analysed by flow cytometry. The immunosuppressive drugs CSA and FK506 suppressed *in vitro* production of the cytokines IFN γ , IL2 and TNF, but not degranulation in pp65-specific CD8 T cells. Both the number of cytokine-producing T cells and the quantity of cytokine secreted per cell were reduced after short-term pre-incubation with CSA or FK506. The cytokine profiles of PBMC incubated with either CSA or FK506 were very similar since both immunosuppressive drugs interfere with the same pathway. CSA and FK506 both inhibit the dephosphorylation of the transcription nuclear factor of activated T cells and thereby inhibit the synthesis of IL2. At physiological concentrations (200 ng of CSA, 10 ng of FK506) the polyfunctional T cell subsets were reduced to approximately the same levels as in transplant patients [265]. In this study the frequencies of CD8 T cells producing

IFN γ , IL2 or TNF as well as the MFIs of IFN γ and TNF were significantly reduced after pre-incubation with immunosuppressive drugs. Engstrand *et al.* (2003), by contrast, reported that the secretion of IFN γ is not affected by *in vitro* incubation with CSA up to 400 ng/ml [199]. In addition, they showed that the MFI of IFN γ did not differ between healthy and immunosuppressed donors. However, Sester *et al.* (2005) showed a decrease in CMV-specific CD8 T cell reactivity at low doses while CD4 T cells were inhibited at higher dosages of CSA and FK506 [146]. Comparably, in the results reported here, the frequency of IFN γ -producing CD8 T cells was downregulated by 50% at concentrations as low as 40ng/ml. In contrast, others found that compared to healthy controls, transplant patients with physiological plasma levels of either CSA or FK506 showed impaired IL2 but not IFN γ -secretion [260].

Investigations with immunosuppressive reagents showed again how important it is to study appropriate markers. If the panel had excluded CD107a, a massive loss of reacting cells would have been seen. By including CD107a it was shown that the T cells were still responding, but the effector mechanisms changed from multiple effector functions to degranulation only. It is possible that these cells produced other effector molecules which were not investigated in this work.

7.6 Outlook

In this study the focus was on the response size rather than donor age, because this seems to be a better indicator of the duration of CMV infection. It certainly appears to be more closely correlated to other observable changes within the T cell response such as subset distribution and polyfunctionality rather than donor age. Since the CMV-specific T cell response size increases over time after CMV infection, for epidemiological reasons, there will be a correlation with age. The data shown here support the idea that the way the immune system responds to CMV by mounting a big or a small response, rather than the infection itself, makes a big change in terms of the effect CMV infection has on the immune system in general.

Many more questions have to be answered, for example, how does CMV accelerate immunosenescence in some people? Why do some people generate large CMV-specific responses while others can control the virus with small numbers of specific T cells? How does CMV induce further differentiated T cells and what role do they play? Why are CD8 T cells more prone to phenotypic changes than CD4 T cells? What are the best biomarkers to measure

immunosenescence? What are the best activation markers to measure CMV-specific T cells? What signalling pathways determine if a T cell is monofunctional or polyfunctional? Do “dysfunctional” T cells exist? What are “protective” T cells? How does CMV alter T cell responses to other antigens?

To help answer these questions, based on the results presented here, this study will be expanded, including more people, especially in the elderly group. To investigate the interaction between CMV and immunosenescence, the immune response to 19 CMV protein-spanning peptide pools will be examined in CMV-seropositive donors. Although pp65 and IE1 are among the major immunodominant proteins, the analysis of more additional proteins might reveal more accurate correlations with age. To get a better picture of the polyfunctional T cell response, the expression of CD107a as degranulation marker will be investigated as well. The memory T cell subsets in these donors will also be analysed to understand how CMV infection contributes to a further advanced memory subset distribution and immunosenescence in some people. These results will hopefully help to better understand the CMV-induced changes in the immune system. Ultimately this will contribute to finding ways of improving the immune response and therefore the quality of life in older people.

8 Appendix

	Stimulation with pp65	Stimulation with IE1	Stimulation with PPD	Stimulation with OKT3
% of all CD4 T cells	Range 0.02-5.80 Median 0.33	Range 0.01-0.4 Median 0.06	Range 0.03-0.72 Median 0.17	Range 8.83-62.41 Median 18.8
Abs numbers of responding CD4 T cells/ml blood	Range 103-13956 Median 2530	Range 53-4616 Median 460	Range 257-5655 Median 1390	Range 19749-410183 Median 156686
% of all CD8 T cells	Range 0.01-11.17 Median 1.28	Range 0.0002-12.13 Median 0.75	Range 0-0.18 Median 0.02	Range 1.79-32.8 Median 8.11
Abs numbers of responding CD8 T cells/ml blood	Range 39-53800 Median 6729	Range 149-40996 Median 5471	Range 11-1578 Median 137	Range 5786-287057 Median 45743

Table 14: Frequencies and absolute numbers of responding T cells after different stimulations.

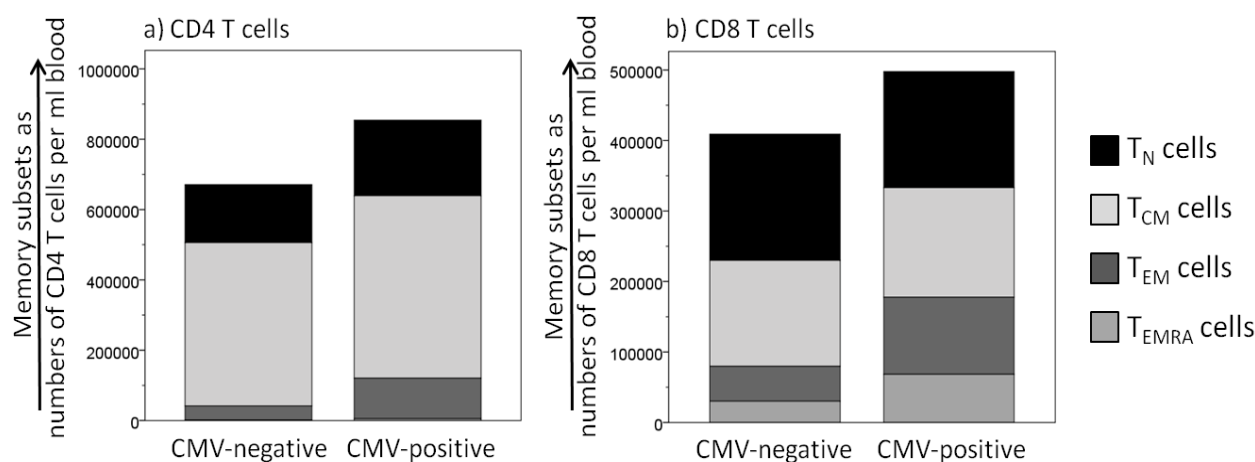


Figure 57: **Distribution of the four memory subsets in all CD4 and CD8 T cells.** Stacked bars display median absolute numbers of the four memory a) CD4 T cell subsets and b) CD8 T cell subsets per ml blood for CMV-negative (n=24) and CMV-positive donors (n=23).

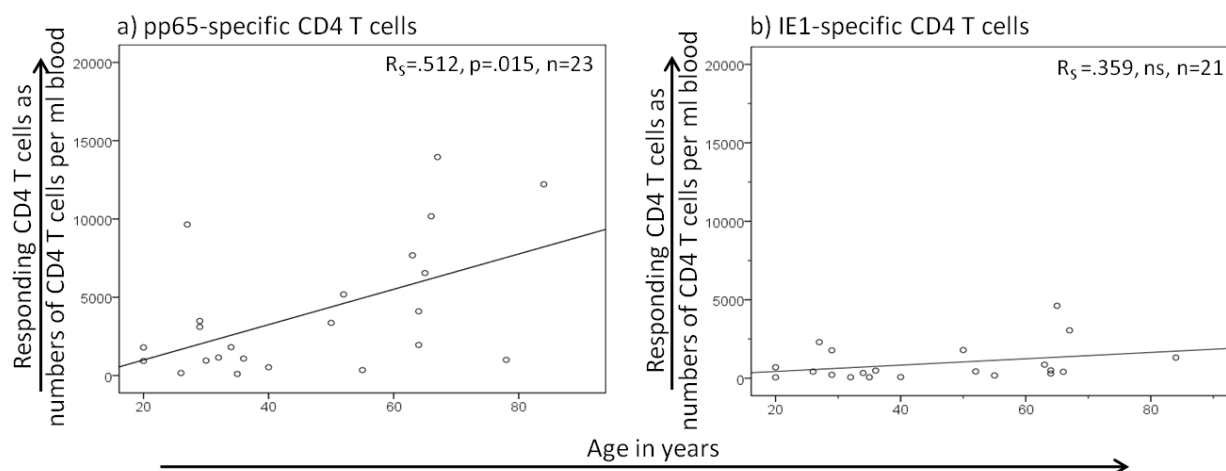


Figure 58: **Absolute numbers of CMV-specific CD4 T cells correlated with age.** Scatter plots show the distribution of frequencies of a) pp65-specific CD4 T cells and b) IE1-specific CD4 T cells and age of the donors.

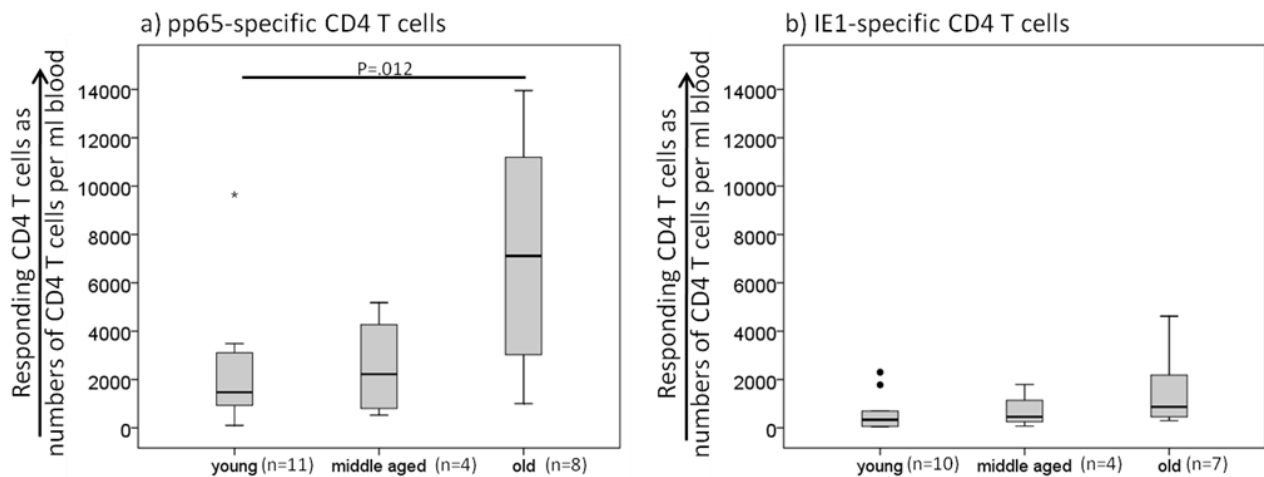


Figure 59: **Absolute numbers of pp65 and IE1-specific CD4 T cells in the three age groups.** Box plots represent absolute numbers of a) pp65-specific CD8 T cells and b) IE1-specific CD8 T cells in the three age groups.

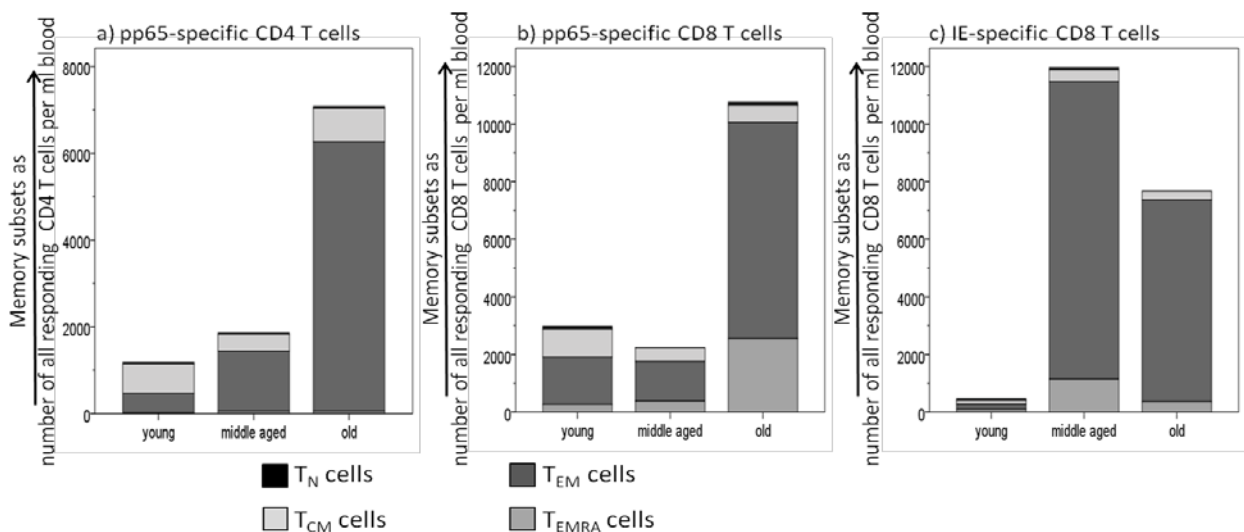


Figure 60: **Distribution of the four memory subsets in CMV-specific T cells in the three age groups.** Stacked bars display median absolute numbers of the four memory T cell subsets for a) pp65-specific CD4 T cells, b) pp65-specific CD8 T cells and c) IE1-specific CD8 T cells in young, middle-aged and old people.

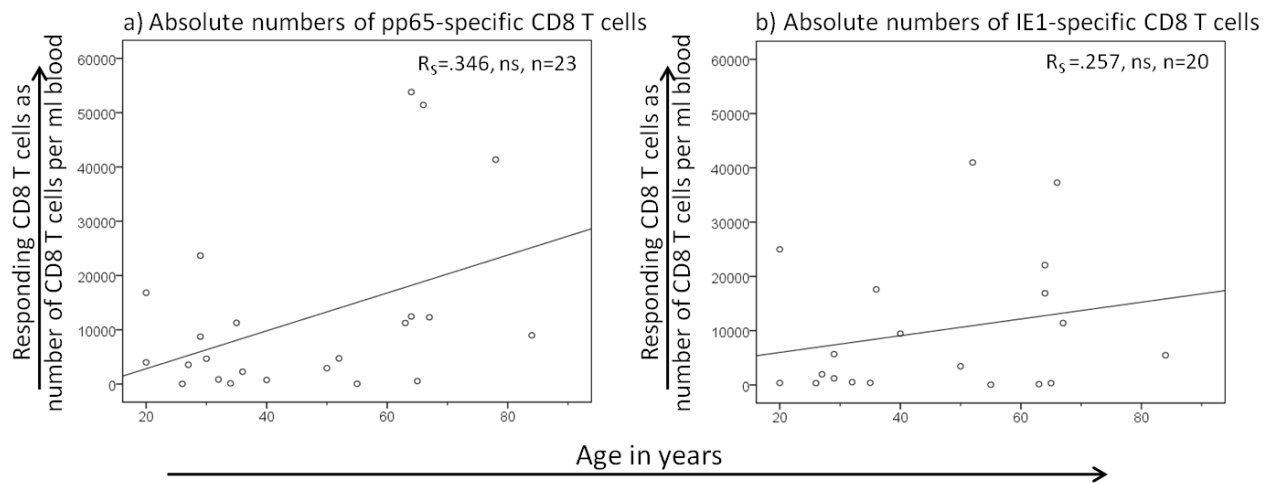


Figure 61: **Absolute numbers of CMV-specific CD8 T cells correlated with age.** Scatter plots show absolute numbers of a) pp65-specific CD8 T cells and b) IE1-specific CD8 T cells and age of the donors.

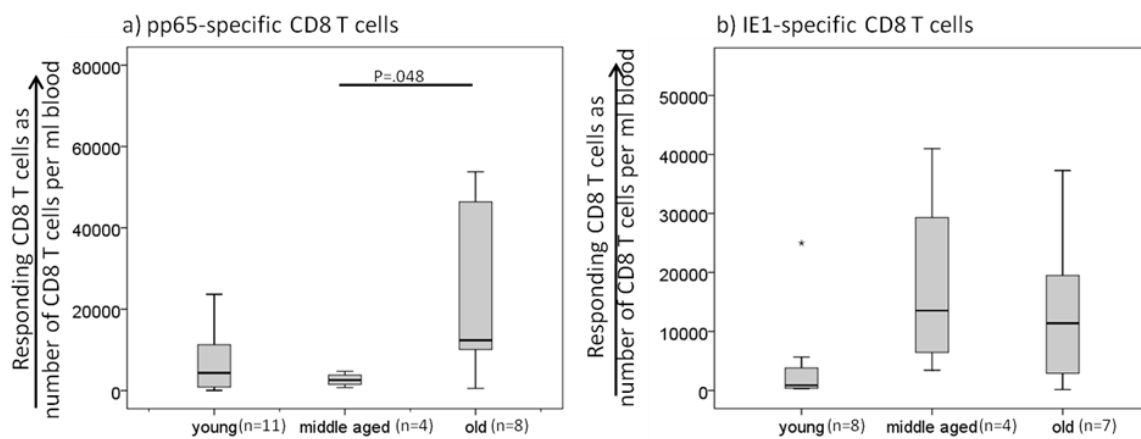


Figure 62: **Absolute numbers of pp65 and IE1-specific CD8 T cells in the three age groups.** Box plots represent absolute numbers of a) pp65-specific CD8 T cells and b) IE1-specific CD8 T cells in the three age groups.

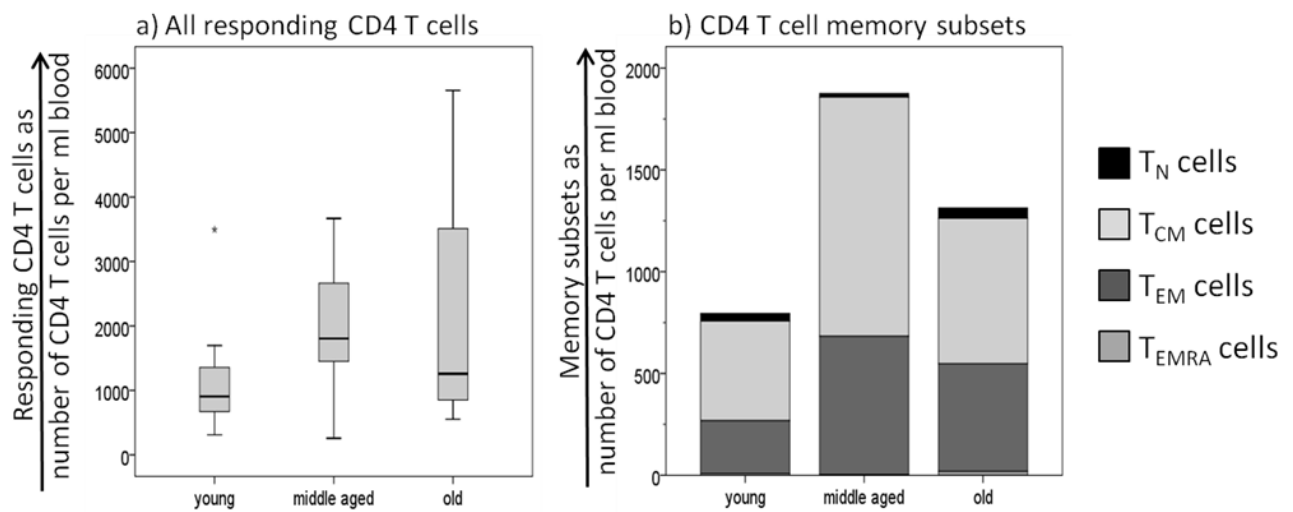


Figure 63: **Absolute counts and memory subsets of responding CD4 T cells in three age groups after PPD stimulation.** a) Box plots represent the absolute numbers of all responding CD4 T cells per ml blood and b) stacked bars display median numbers of the four memory subsets in responding CD4 T cells after stimulation with PPD in young (n=11), middle-aged (n=9) and old (n=4) donors.

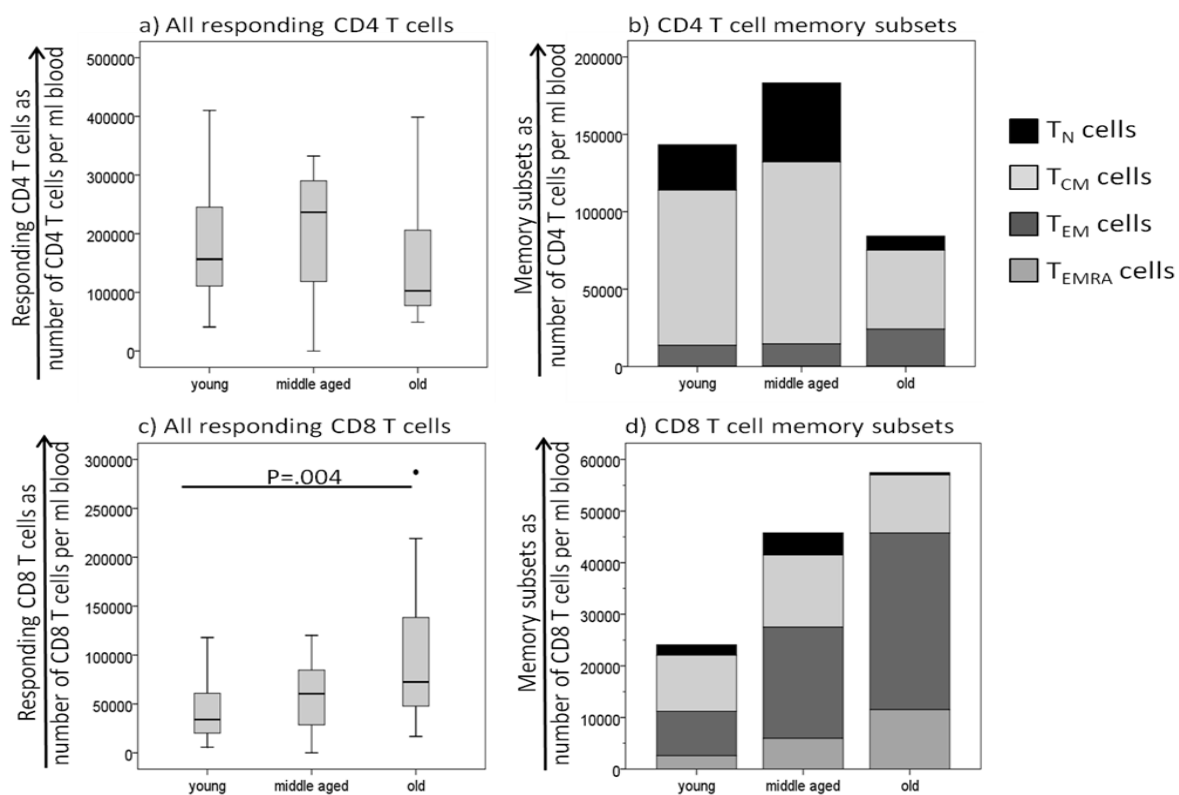


Figure 64: **Absolute counts and memory subsets of responding CD4 and CD8 T cells in three age groups after OKT3 stimulation.** Box plots represent the absolute numbers of all responding a) CD4 and c) CD8 T cells per ml blood and stacked bars display median numbers of the four memory subsets in responding b) CD4 and d) CD8 T cells after stimulation with OKT3 in young (n=24), middle-aged (n=12) and old (n=12) donors.

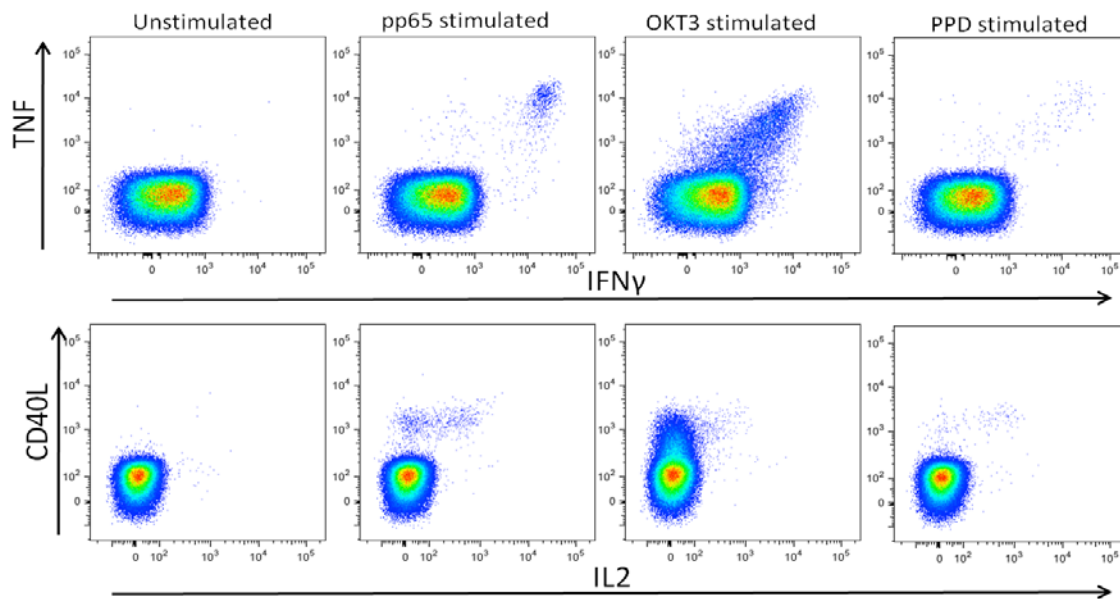


Figure 65: **Representative dot plots from one old donor show the distribution of CD4 T cells after different stimulations.** Upper panels show TNF plotted against IFN γ , lower panels show CD40L plotted against IL2. Shown are from left to right unstimulated, pp65 stimulated, OKT3 and PPD stimulated CD4 T cells.

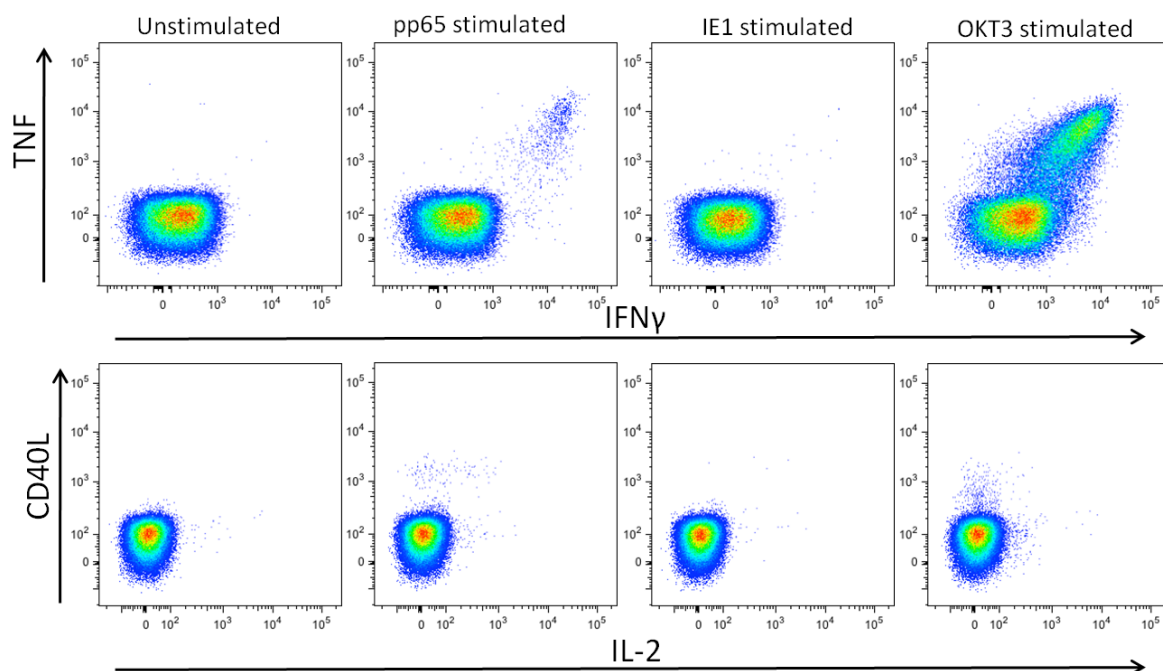


Figure 66: **Representative dot plots from one old donor show the distribution of CD8 T cells after different stimulations.** Upper panels show TNF plotted against IFN γ , lower panels show CD40L plotted against IL2. Shown are from left to right unstimulated, pp65 stimulated, IE1 stimulated and OKT3 stimulated CD8 T cells.

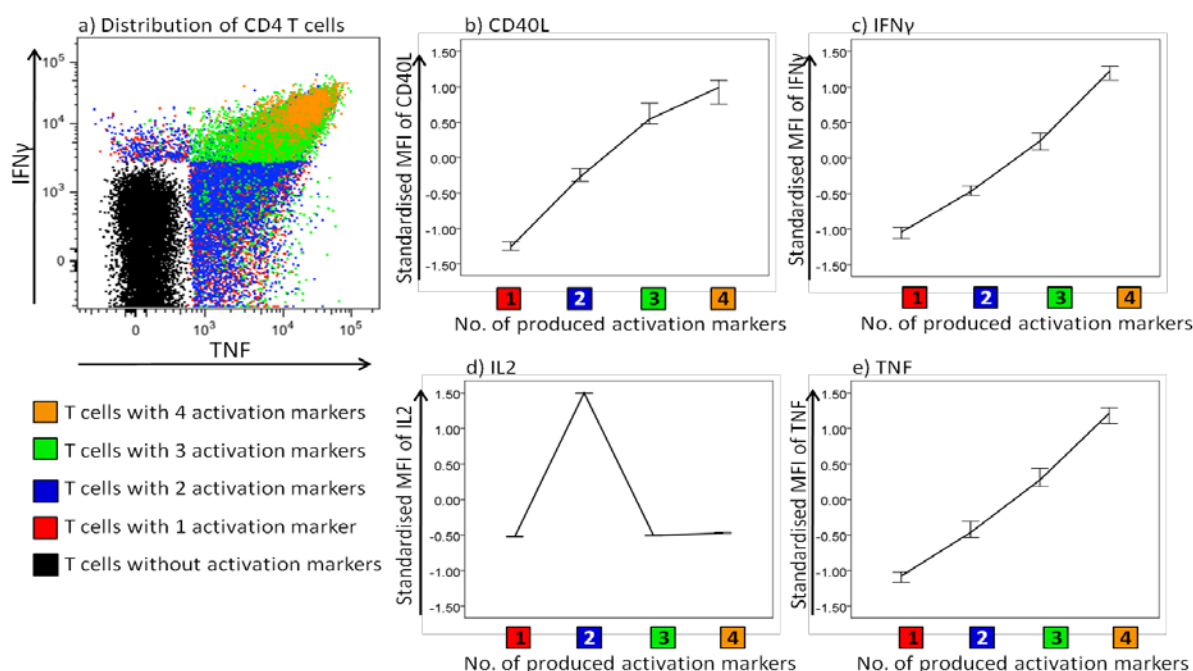


Figure 67: **Expression of activation markers in polyclonally stimulated CD4 T cells.** Representative dot plot shows the distribution of CD4 T cells after stimulation with OKT3 for 16 hours. Line curves show standardised MFIs of a) IFN γ , b) IL2, c) TNF and d) CD40L in CD4 T cells expressing one (red), two (blue), three (green) or four (orange) activation markers (n=28).

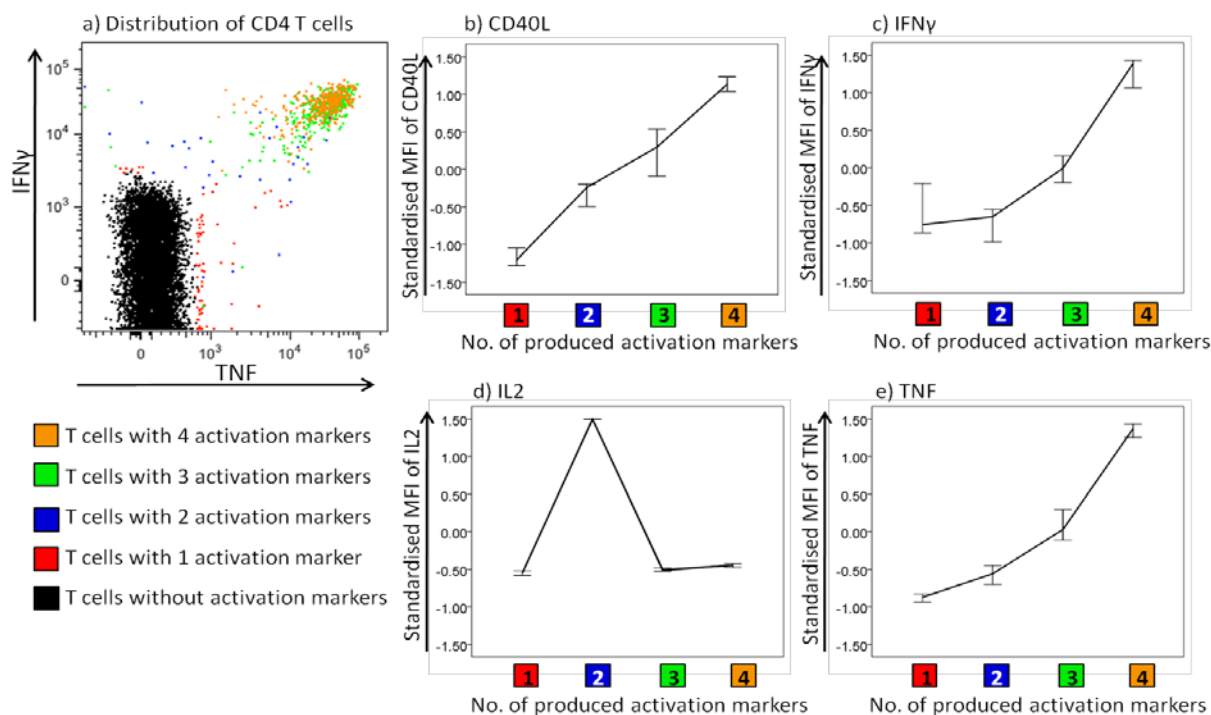


Figure 68: **Expression of activation markers in PPD-specific CD4 T cells.** A) Representative dot plot shows the distribution of CD4 T cells after stimulation with PPD for 16 hours. Line curves show standardized MFIs of b) IFN γ , c) IL2, d) TNF and e) CD40L of CD8 T cells expressing one (red), two (blue), three (green) or four (orange) activation markers (n=16).

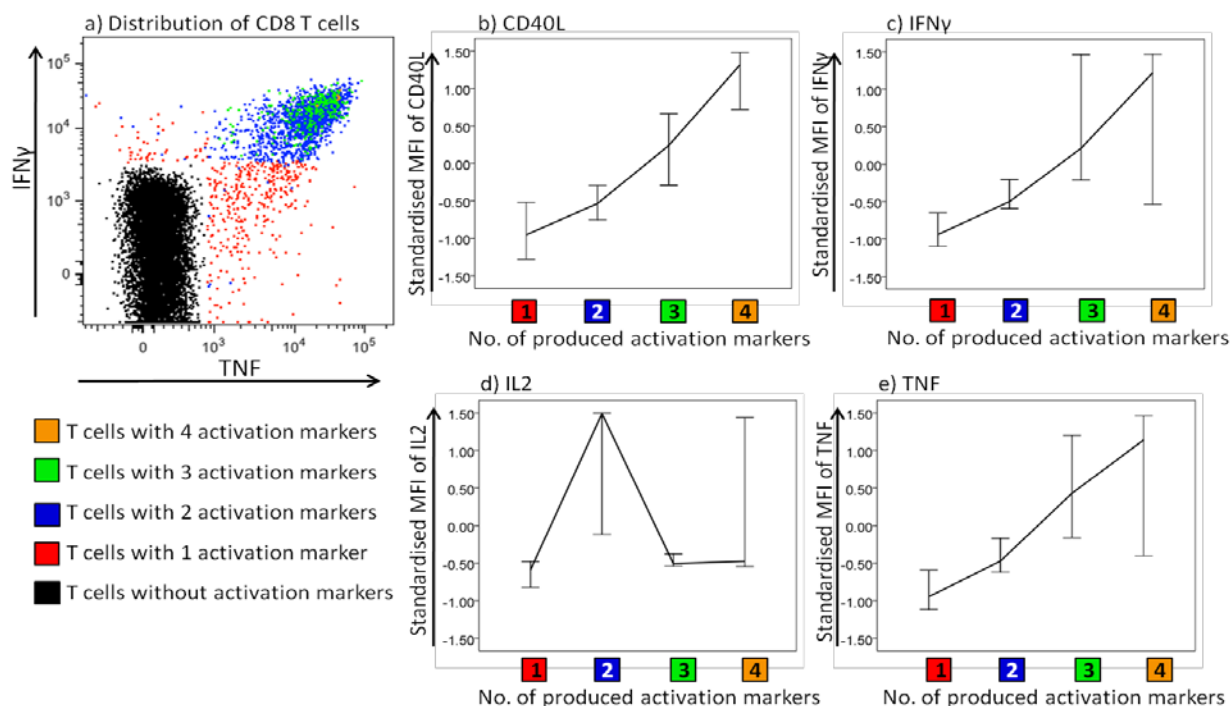


Figure 69: **Expression of activation markers in IE1-specific CD8 T cells.** Representative dot plot shows the distribution of IE1-specific CD8 T cells after stimulation with IE1. Line curves show standardised MFIs of a) IFN γ , b) IL2, c) TNF and d) CD40L in CD8 T cells expressing one (red), two (blue), three (green) or four (orange) activation markers (n=10).

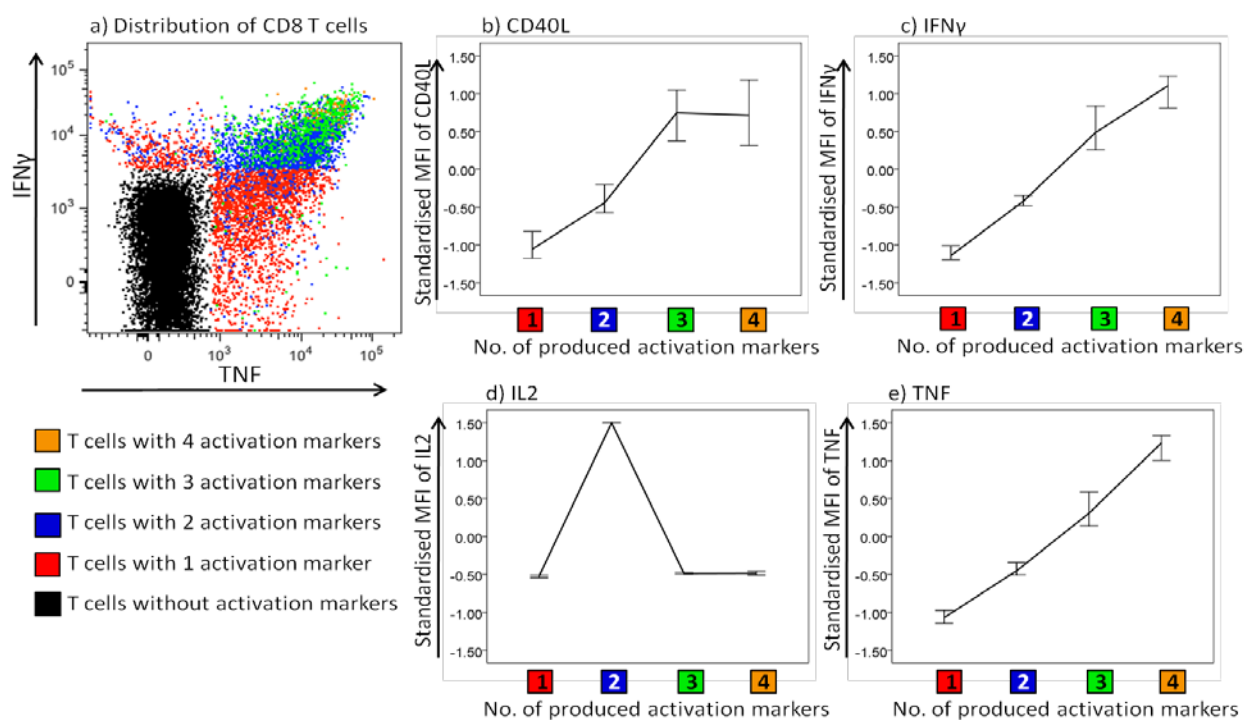


Figure 70: **Expression of activation markers in polyclonally stimulated CD8 T cells.** a) Representative dot plot shows the distribution of CD8 T cells after stimulation with OKT3 for 16 hours. Line curves show standardised MFIs of b) IFN γ , c) IL2, d) TNF and e) CD40L in CD8 T cells expressing one (red), two (blue), three (green) or four (orange) activation markers (n=28).

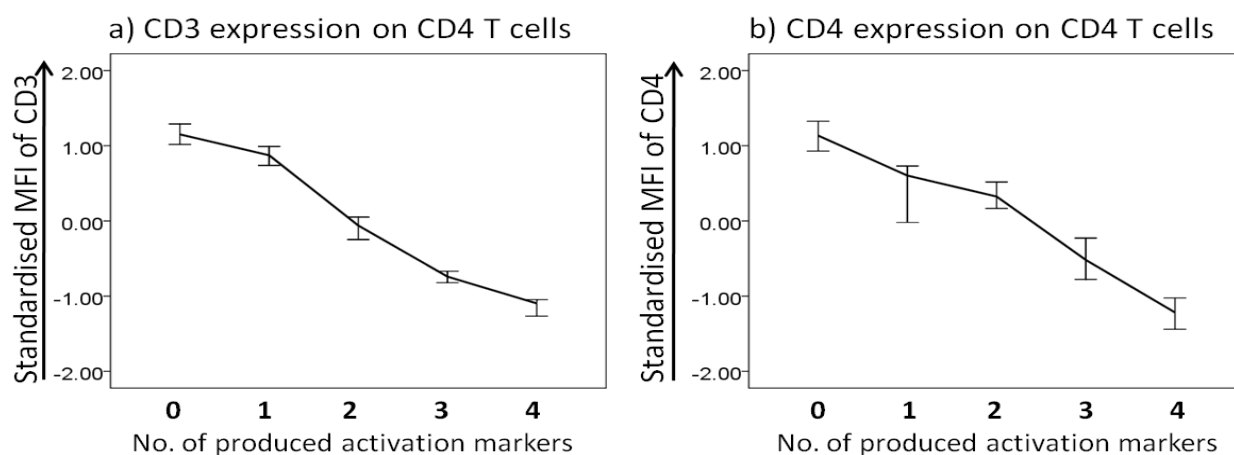


Figure 71: **Downregulation of CD3 and CD4 on CD4 T cells after PPD-stimulation.** Line curves represent standardised MFIs of a) CD3 and b) CD4 expression on CD4 T cells after PPD-stimulation expressing different numbers of markers (n=16).

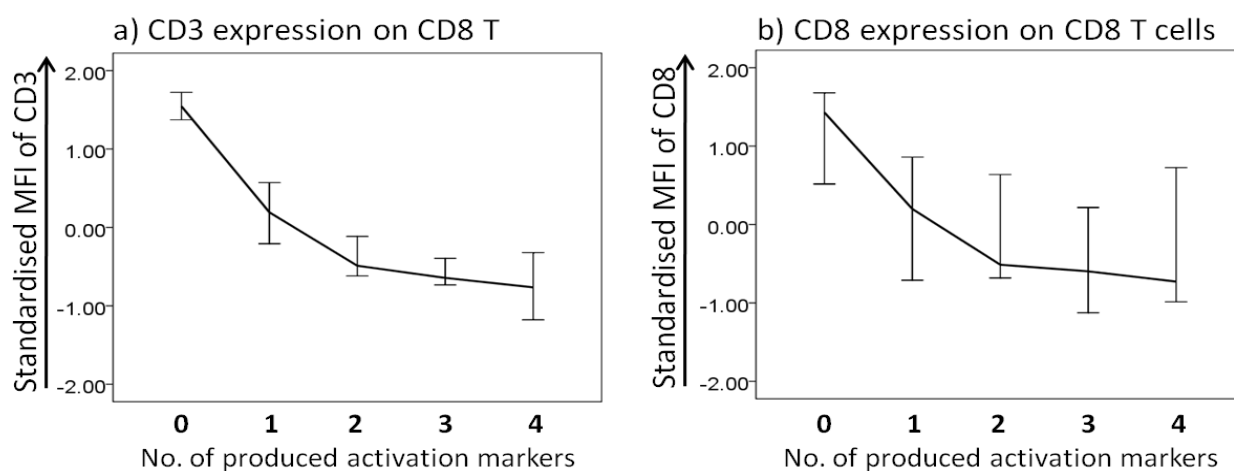
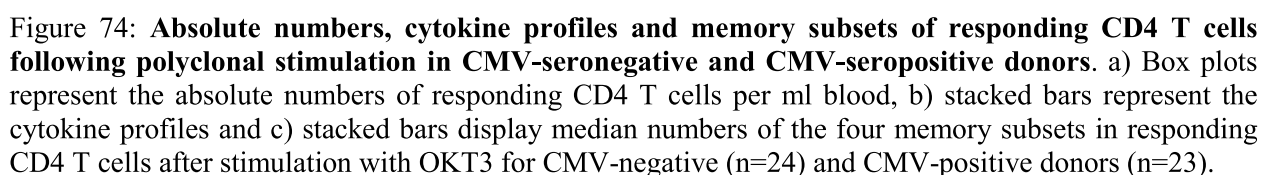
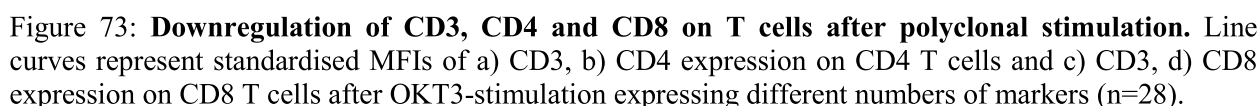


Figure 72: **Downregulation of CD3 and CD8 on CD8 T cells after IE1-specific stimulation.** Line curves represent standardised MFIs of a) CD3 and b) CD8 expression on IE1-specific CD8 T cells expressing different numbers of markers (n=10).



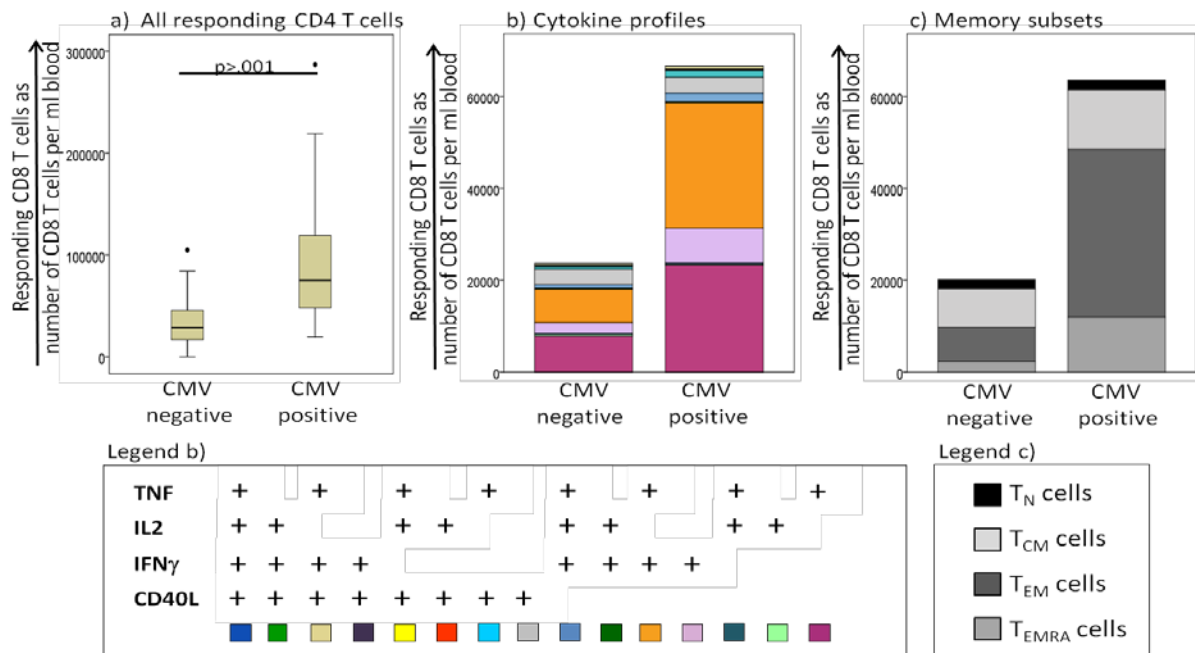


Figure 75: **Absolute numbers, cytokine profiles and memory subsets of responding CD8 T cells following polyclonal stimulation in CMV-seronegative and CMV-seropositive donors.** a) Box plots represent the absolute numbers of responding CD8 T cells per ml blood, b) stacked bars represent the cytokine profiles and c) stacked bars display median numbers of the four memory subsets in responding CD8 T cells after stimulation with OKT3 for CMV-negative (n=24) and CMV-positive donors (n=23).

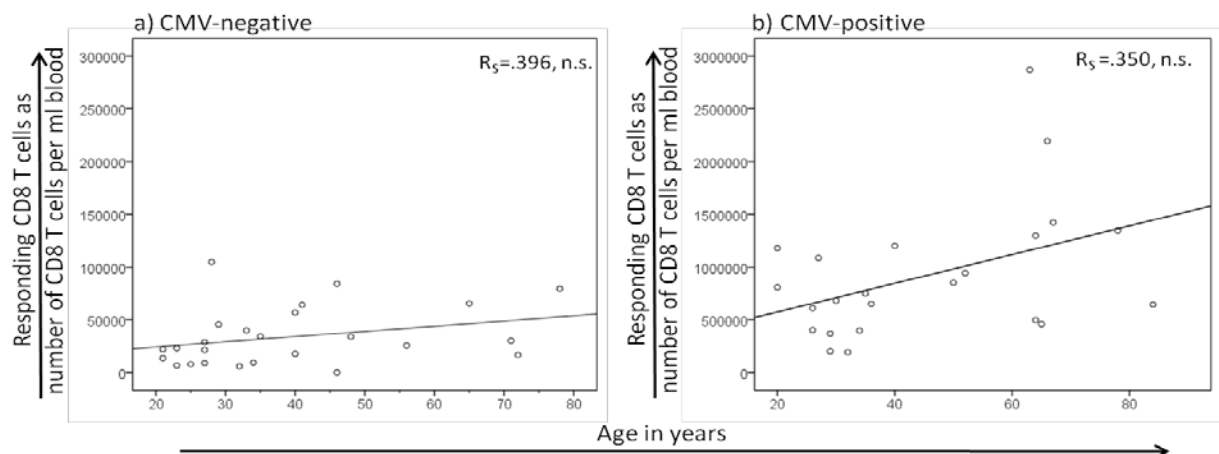
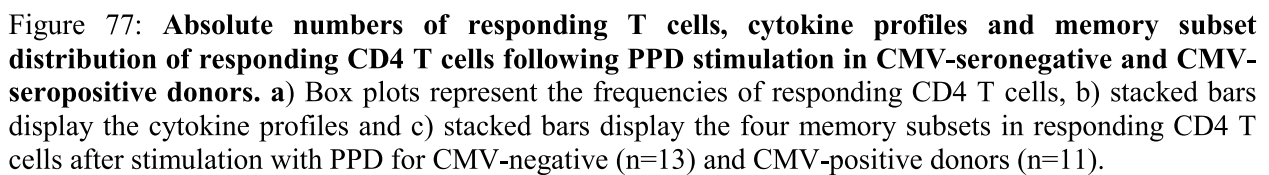


Figure 76: **Correlation of CMV status, age and absolute response size after OKT3 stimulation.** Scatter plots represent the number of responding CD8 T cells after stimulation with OKT3 in a) CMV-negative (n=24) and b) CMV-positive (n=23) donors related to age of the donor.



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265. Fuhrmann S, L.R., Ganepola S, Bold G, Reinke P, Lehmkuhl, H, Hetzer, R, Uharek, L, Volk H Kern F, *Post-transplantation immunosuppression results in loss of T-cell polyfunctionality* Exp Med, submitted

10 Publications and presentations

Publications

Dissection of the CMV specific T-cell response is required for optimized cardiac transplant monitoring.

Kirchner A, Hoffmeister B, Cherepnev-G G, Fuhrmann S, Streitz M, Lachmann R, Bunde T, Meij P, Schönemann C, Hetzer R, Lehmkuhl HB, Volkmer-Engert R, Volk HD, Gratama JW, Kern F.

J Med Virol. 2008 Sep;80(9):1604-14

Simultaneous characterization of phospho-proteins and cell cycle in activated T cell subsets.

Lanuti P, Fuhrmann S, Lachmann R, Marchisio M, Miscia S, Kern F.

Int J Immunopathol Pharmacol. 2009 Jul-Sep;22(3):689-98

Post-transplantation immunosuppression results in loss of T-cell polyfunctionality

Fuhrmann S, Lachmann R, Ganepola S, Bold G, Reinke P, Lehmkuhl, H, Hetzer, R, Uharek, L, Volk H Kern F.

Exp Med, submitted

Phospho-PKC α in A β 1-42-activated human T cells discriminate Alzheimer's disease from Lewy body dementia

Lanuti P, Ciccocioppo F, Bonanni L, Marchisio M, Lachmann R, Tabet N, Pierdomenico L, Santavenere E, Catinella V, Iacone A, Thomas A, Gambi D, Miscia S, Onofri M, Kern F

JIC, submitted

IL-7 Induces Short-Lived, Multifunctional CD4⁺ CD27-CD45RA⁺ T Cells That Accumulate During Persistent Cytomegalovirus Infection

Libri V, Azevedo R, Jackson S, Di Mitri D, Lachmann R, Fuhrmann S, Vukmanovic-Stejić M, Yong K, Battistini L, Kern F, Soares M, Akbar A.

Blood, submitted

Polyfunctional T-cells accumulate in large Cytomegalovirus-specific T-cell responses

Lachmann R, Akbar A, Kern F

Blood, submitted

Presentations

- 2006 Talk: Tagung der Deutschen Parasitologischen Gesellschaft
- 2006 Poster: 2nd MASIR Conference Measuring Antigen-Specific Immune Responses
- 2009 Poster: BSMS Neuroscience research day (Winner of poster prize)
- 2010 Poster: 4th MASIR Conference Measuring Antigen-Specific Immune Responses
- 2010 Poster: 14th International Congress of Immunology